THE PHYSIOLOGY OF FUNGI AND FUNGUS DISEASES

PAPERS PRESENTED ON THE OCCASION OF THE 50TH ANNIVERSARY OF THE ESTABLISHMENT OF THE DEPARTMENT OF PLANT PATHOLOGY AT WEST VIRGINIA UNIVERSITY 1912-1962

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WEST VIRGINIA UNIVERSITY AGRICULTURAL EXPERIMENT STATION

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1912-1962

WEST VIRGINIA UNIVERSITY AGRICULTURAL EXPERIMENT STATION

West Virginia University
Agricultural Experiment Station
College of Agriculture, Forestry, and Home Economics
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Morgantown

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Introduction

THE Department of Plant Pathology was officially recognized as a separate department of the Agricultural Experiment Station of West Virginia University on July 1, 1912. In the 50 years that have elapsed since this event, the Department has made many important contributions to the science of plant pathology. Especially noteworthy is the pioneer and basic research on the physiology of fungi and the diseases they cause.

When it was decided to observe the 50th Anniversary of the establishment of the department, it seemed fitting that emphasis be placed on this subject. With this in mind, Dr. J. C. Walker, Professor of Plant Pathology at the University of Wisconsin, was invited to present a lecture on the physiology of disease resistance, a field in which he has made outstanding contributions.

To aid us in our birthday celebration, the Potomac Division of the American Phytopathological Society was invited to hold its annual meeting at West Virginia University in Morgantown. Our invitation was accepted. In recognition of the anniversary, the program was arranged to include a symposium on "Disease and Fungus Physiology" in which invitation papers were presented by Dr. V. G. Lilly, Professor of Physiology, and Dr. H. L. Barnett, Professor of Mycology, at West Virginia University, and Dr. A. E. Dimond, Chief of the Department of Plant Pathology of the Connecticut Agricultural Experiment Station.

The anniversary address by Dr. Walker and the three symposium papers are presented in this bulletin.

The Physiology of Disease Resistance

Anniversary Address
Monday, April 23, 1962

J. C. Walker
Professor of Plant Pathology
University of Wisconsin

FIFTY years ago when this department was being organized, plant pathology in this country was at the threshold of a half-century of outstanding growth. There were many acute plant disease problems which required immediate attention. Some diseases, such as cereal smuts, apple scab, and potato late blight, were coming under control by the use of fungicides, but others, such as cotton wilt, flax wilt, tomato wilt, cabbage yellows, and wheat rusts, were not destined to yield to this approach. It was with this latter group of diseases, therefore, that the development of resistant varieties gained its greatest momentum. Within the past five decades these and many other diseases have come under partial or complete control by this means. Our food, feed, and fiber supplies are abundant today, in part at least, because of the persistent efforts of plant breeders and plant pathologists.

The development of a resistant variety is not an overnight matter. It usually requires many years before resistance is combined with satisfactory quality, yield, and other desirable characters. Investigators in this area soon learned of difficulties and obstacles along this path of slow progress. One of these was, as we all well know, the variability of the pathogenic entity—fungus, bacterium or virus. While man is speeding up nature's plan of survival of the fittest among the crop plants, nature is also helping pathogenic agents to survive by the same means. Some pathogens are more cantankerous than others in this regard. Some skeptics fifty years ago prophesied that we were wasting time in trying to keep ahead of the variable pathogens. To some degree they were right; in some respects they were entirely wrong. The best things that have come out of these fifty years of experience are not the resistant varieties that add to our ease of living, but rather the realization of the fact that the more we know about the underlying fundamentals of variability the more likely we are to succeed, and the more permanent may be our success.

The entrance of modern genetics into the field of plant breeding was a fortunate accident for plant pathology. W. A. Orton (30) believed he was dealing with a Mendelian character in resistances to wilts of cotton, cowpea, and watermelon even though he did not attempt to fit his data to a factorial hypothesis. Biffen (6, 7), in the case of resistance in wheat to stripe rust (*Puccinia glumarum* [Schm.] Eriks. and Henn.), was the first to do this. His results and those of others which followed with rusts were clouded or confused by mixtures of pathogenic races. Clean-cut evidence of Mendelian inheritance of resistance to well-defined races of the pathogen came with Aamodt's (1) work on black stem rust of wheat (*Puccinia graminis tritici* [Eriks. and Henn.]) in 1923, and the work of Burkholder (9, 10) and of McRostie (26) with resistance to three races of bean anthracnose organism (*Colletotrichum lindemuthianum* [Sacc. and Magn.] Scribner) (1918-1923). Since these early days the inheritance of resistance has been worked out for many diseases, particularly where stable, well-defined races of the pathogen are available. While it is not my purpose to dwell here on the genetics of disease resistance, it is well to point out certain principles which have become established over the past fifty years.

As with other plant characters, resistance may be controlled by a single gene pair or a series of alleles at a given locus. There may be distinct genes at different loci, each of which may independently control resistance, as in powdery mildew of barley and angular leaf spot of cotton. Resistance or susceptibility may be controlled by the complementary action of two duplicate gene pairs as in alfalfa mosiac of bean or by three gene pairs, as in onion smudge. It may be due to a dominant inhibitor gene which prevents the expression of an otherwise functional susceptible gene, e.g., common bean mosaic (2), or an inhibitor which prevents the expression of a resistant gene, e.g., onion smudge (48). The degree of resistance controlled by a major gene pair may be increased by the reinforcement of an undefined number of modifying genes, e.g., cucumber mosaic (49). These examples so far might all be classed as qualitative characters in that fairly well defined discontinuous resistant and susceptible classes can be set up for plants in segregating progenies from crosses between resistant and susceptible parents. There are in contrast many examples in which it is impossible to set up discontinuous resistant and susceptible classes within segregating progenies. In such cases inheritance is quantitative in character and many genes are involved. Their effects are additive and presumably are not dependent upon a single gene pair for their expression. There is nothing unique about the Mendelian character of

these various types of resistance; the same range may be found among other plant characters. The important thing about it for our present discussion is that the worthwhile disease resistant characters are Mendelian. It is necessary to keep this fact in the forefront not only in the development of resistant varieties, but also in any study of the physiology of host resistance.

While our science has accumulated a store of information on the heredity of resistance of host plants, it has, though more tardily, been accumulating basic information on variability of pathogens. We are concerned here primarily with variability in pathogenicity. When these differences are determined on the basis of resistance or susceptibility of a given host variety, species (or group of species or varieties), I prefer to speak of the character as selective pathogenicity. Races of a fungus or bacterium which are distinguished on this basis I am referring to as pathogenic races; that is, we are concerned with types of physiologic races which are defined on the basis of pathogenicity rather than on any other of a number of definite or indefinite physiologic characters. I prefer this to the more recently introduced terms "virulent" and "avirulent." Virulence has long been used in our literature to refer to degree of pathogenic action rather than selective pathogenicity. Anyone who has worked with facultative parasites knows that it is not uncommon to find isolates within a species or race which differ in their virulence, i.e., in rate and degree of pathogenic action on a given host species or variety. They may not differ, however, in their selective pathogenicity. In the evaluation of host resistance it is necessary to distinguish between these two types of pathogenicity. I shall be talking principally about selective pathogenicity in this discussion.

The distinction of pathogenic races is based necessarily on the resistance or susceptibility of the host. Well-defined races are most common among the obligate parasites, especially the rusts and powdery mildews. In the course of evolution it is quite likely that these pathogens have lost the attributes of their ancestors which were able to survive and thrive on relatively simple organic and inorganic foodstuffs. In this evolutionary trend they have come to depend on various highly organized compounds which their ancestors or less particular relatives may use, but do not need. In fact, they are so particular that they not only require the metabolites of living host cells, but also the metabolites of certain varieties of a given host species. With this great loss of dietary versatility the rusts and mildews would probably have long since disappeared in the competitive struggle of evolutionary processes had they not retained and selected out a highly efficient gene-controlled

mechanism whereby they could shift their selective attack as their substrates in the form of susceptible varieties were eliminated. Thus, with regard to this unique mechanism of variability, they are at the top of the evolutionary ladder among fungi, but as to their ability to exist on relatively simple food substrates they are at the very bottom of the ladder.

The genetics of pathogenicity has come into its own more recently than the genetics of host resistance. The concept of mutation in microorganisms was accepted more slowly. Moreover, the recognition of heterothallism in various groups of fungi was not taken up as rapidly as it might have been as an essential tool for the study of hereditary characters. But in the past 20 years it has become well established that pathogenic characters in microorganisms are gene controlled, just as resistant characters to such pathogens are gene controlled. Variability within a pathogenic species is based first upon mutation, and new races commonly appear as the result of recombination of genes through the normal sexual processes. Since many pathogenic fungi have lost or make little use of a sexual stage such recombination would be impossible were it not for the phenomena of heterocaryosis and parasexualism, which provide means for the appearance of new races without the intervention of an orthodox sexual stage. While the data accumulated thus far on the inheritance of pathogenicity are less numerous than those for inheritance of resistance, it is evident that the same genetic range is to be found. In the case of flax rust pathogenicity and resistance are each controlled by specific genes with the result that resistant and susceptible classes of the host as well as pathogenic and nonpathogenic races of the fungus are quite easily defined. In other words, the pathogenicity of a given race to a given host variety may be controlled by a single gene pair in each, and few, if any, modifiers are concerned in either host or pathogen. This rather simple situation led Flor (17) to propose his hypothesis of a "gene-to-gene" relationship between pathogenicity and resistance. In other rust diseases, black stem rust, for instance, where intermediate degrees of resistance and pathogenicity occur, a superficial examination would suggest that the existence of rather influential modifiers either in the host or in the pathogen or in both will make the genetic interpretation of resistance and pathogenicity by a gene-to-gene hypothesis much more difficult. Still more confused are such extreme cases as the Rhizoctonia disease where variation in selective pathogenicity and in host resistance would both appear to be multigenic.

The recognition of the genetic bases of resistance and pathogenicity is important to an inquiry into and an understanding of the physiology

of disease resistance. It is essential at the outset to distinguish between variability due to simple or complex inherited mechanisms and that due to the play of environmental factors upon a genetically stable host population. This was the basis of the differences in opinion fifty years ago between Biffen (6, 7), the geneticist, on the one hand, and Butler (11) and Pole-Evans (31), the pathologists, on the other. The first considerations of the physiology of resistance came from observations on the effect of the various environal factors on the expression of this character.

My own first experience in this area was with cabbage yellows (*Fusarium oxysporum f. conglutinans* [Wr.] Snyder and Hansen). This disease was one of the first to be studied with respect to the relation of soil temperature to disease development. It was shown to be restricted or even prevented by low soil temperature and to have an optimum constant soil temperature of about 28°. When Jones and Gilman (24) developed yellows resistant Wisconsin Hollander by mass selection they made selections of mature plants and tested the progenies with plants well past the early seedling stage. When Tisdale (40) and later Tims (39) studied resistant Wisconsin Hollander in comparison with susceptible Hollander in soil temperature tanks in the early seedling stage they found the striking field resistance of Wisconsin Hollander to be almost absent. This is one of the best illustrations of the effect of environment on disease resistance, and also of the development of the resistant character during growth of the host. Some years later, when through inbreeding and more rigorous selection a much higher type of resistance was obtained (44), it was shown to be highly effective from the time of seed germination on, and to withstand the effect of soil temperature near the optimum for the disease (47). The two types of physiological response to resistance suggested that they might be different genetically. This was eventually shown to be the case (4, 8). The second (more stable) type of resistance (Type A) was shown to be monogenic and dominant. The first type (that of Wisconsin Hollander) (Type B) was shown to be multigenic and only partially dominant. Thus, these two types of resistance in the same host to the same race of the pathogen are distinct physiologically and genetically. There are probably many factors other than temperature involved in this difference, but the difference is a very convenient one to use in various ways. For instance, it has been shown that in Type A resistant plants the dominant allele (*R*) determines resistance in the heterozygous (*Rr*) or homozygous (*RR*) state as opposed to the homozygous (*rr*) genotype of the susceptible line. It can also be shown by manipulation of soil temperature that Type B resistant plants, e.g., Wisconsin Hol-

lander, are also homozygous for the susceptible allele (*rr*) and that their resistance therefore is dependent upon the repressive effect of other genes on the influence of this susceptible allele. Genetic and physiological studies combined showed that different levels of resistance could be attained by selection. In the early seedling stage these levels could be determined by manipulating soil temperature. Apparently the larger the number of genes, the higher the soil temperature needed to break down resistance. However, at 24° the effects of the Type B genes on resistance were completely suppressed, while the function of the Type A gene was still complete. This is the basis for the current standard procedure for testing breeding progenies and commercial varieties for Type A resistance.

Further evidence that the level of Type B resistance in a given variety is the function of the level of secondary genes accumulated by selection can be shown by use of the physiological approach. Wisconsin Hollander and Bugner are Type B varieties derived from the same basic stock, but the former has been selected more rigidly than the latter and in consequence has a higher level of resistance. Badger Ballhead is homozygous for the Type A gene and is also derived from the same stock. When these are compared in the early seedling stage with each other and with a standard susceptible from the same stock (in this case Oakview Ballhead) at different soil temperatures applied at different time intervals, the physiological response of resistance to environment is clearly shown (Figure 1). In all cases the seedlings were grown in quartz sand to the cotyledon stage when the roots were dipped in a spore suspension of the pathogen, replanted in sand, and watered with Hoagland nutrient solution. In Figure 1, A are seedlings which have been exposed for 28 days after inoculation at a constant soil temperature of 20°, which is considerably below the optimum for the disease, but probably near the equivalent of diurnal temperatures of soil in the field at the time of the year when yellows is most destructive. The early seedling picture here corresponds closely to that of the average field picture, where all three resistant varieties are standing up while the susceptible variety has succumbed. In Figure 1, B, a similar set of seedlings is shown. This set was exposed at a temperature of 24° for only 14 days. This is roughly equivalent to the field picture in an unusually warm summer. The Type A resistant line is standing up perfectly, but Type B resistant varieties are not. The physiological effect of the warmer temperature is also bringing out a distinction between the two Type B varieties. In Wisconsin Hollander the effect of more rigid selection over a long period of years

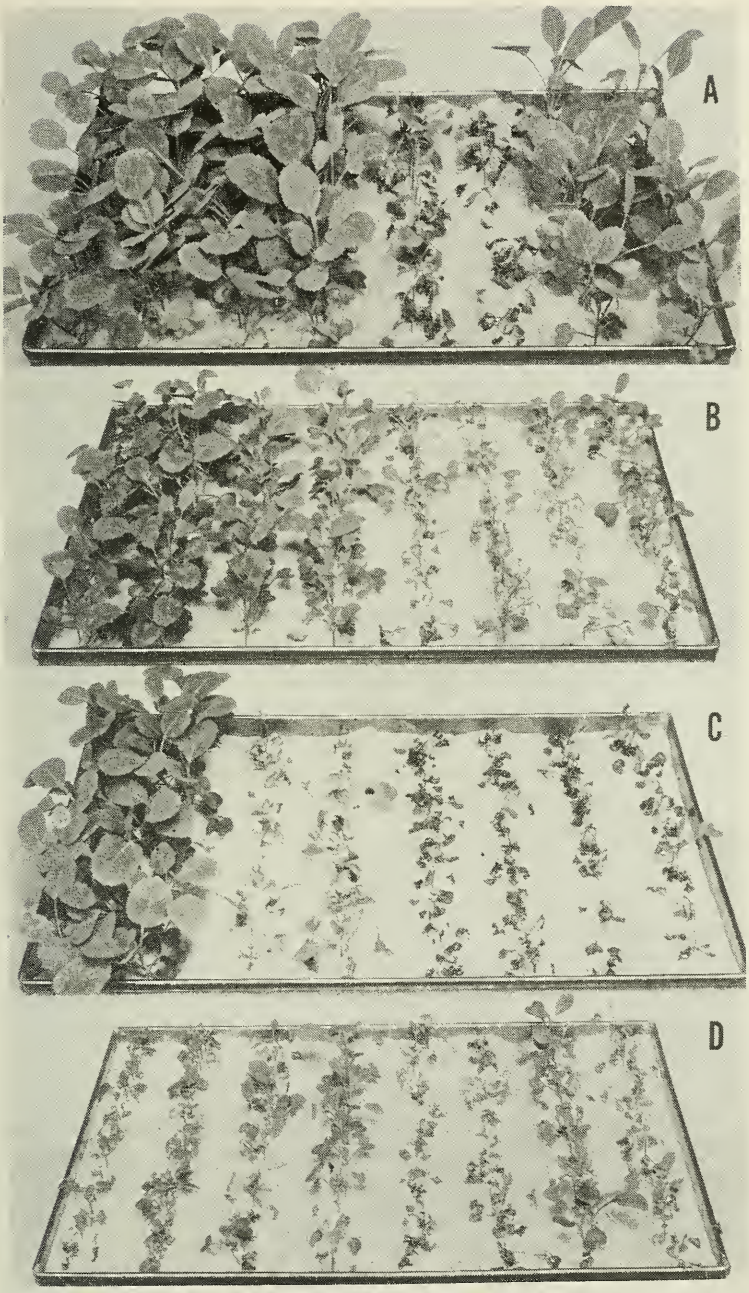

FIGURE I. Relation of soil temperature to expression of monogenic (Type A) and multigenic (Type B) resistance to yellows in cabbage seedlings. The lots were exposed after inoculation as follows: A. 20° C for 28 days; B. 24° for 14 days; C. 24° for 21 days; D. 28° for 14 days. See text for further details.

has resulted in a higher degree of resistance than that of Bugner. It is probable that in the former a larger number of modifying genes have been accumulated which collectively suppress the effect of the homozygous recessive susceptible allele. In Figure 1, C the same set of plants is shown seven days later. By this time the effect of all the modifying genes in Wisconsin Hollander and Bugner has been completely suppressed. This is a condition which never exists in the field. It provides a technique, however, useful in breeding procedures in which Type A and Type B resistant individuals can be separated with certainty in the early seedling stage, a distinction which cannot be made in the field. This series of comparisons indicates quite clearly that these two types of resistance in the same plant act differently physiologically at different soil temperature levels. Since they are known to be distinct genetically, it can safely be assumed that there are different gene-controlled processes in the two types. It is also clear that the single-gene process is the most stable and least responsive to change in temperature. In Type B resistance, if there are many gene-controlled processes involved, they probably each respond differently to temperature and their combined effect on resistance is effective only at the lower part of the temperature range. The final act in this drama is shown in Figure 1, D. Here young seedlings were exposed at 28°, the optimum for disease development, for 14 days. This shows that even the "stable" monogenic resistance can be broken down completely under this set of conditions.

The same physiological response of resistance to Fusarium wilt (*Fusarium oxysporum* f. *lycopersici* [Sacc.] Snyder and Hansen) has been shown in tomato when monogenic resistant, e.g., Jefferson, and multigenic resistant, e.g., Marglobe, varieties were compared. It is probably common to other vascular fusarial wilts and to many other diseases. In the cereal rusts, for instance, varieties highly resistant to a given race or group of races may "switch" to the susceptible class at extremely high temperature. The same type of response seems to hold when varieties of potato resistant to *Phytophthora infestans* (Mont.) DeBy. are compared. Here we have monogenic resistance to a given race or to several races on the one hand and multigenic resistance effective against most, if not all, races on the other hand. Again monogenic resistance is more stable under a wider range of environment than is multigenic resistance (18).

Another means whereby the physiology of resistance has been studied is through the effect of host nutrition on its expression. Many years ago Hassebrauk (21) in Germany studied the effect of various

nutrients fed the wheat plant on the expression of resistance to several cereal rusts. He found that highly resistant varieties were little affected by variation of level and/or concentration of major elements in the nutrient, while in varieties intermediate between high resistance and extreme susceptibility the expression of resistance could be shifted over a certain range by variation in the nutrient balance. We have studied resistance to cabbage yellows (46) and tomato Fusarium wilt (45) by this approach. These two diseases respond quite similarly to variation in host nutrients. In the susceptible varieties the diseases are suppressed with increases in salt concentration and osmotic density of the nutrient solution. They are enhanced by high levels of nitrogen and low levels of potassium. Again a marked difference in the response of multigenic and monogenic resistance to nutrition is evident, as shown in Figure 2. With increase in salt concentration of the nutrient solution the downward trend of the disease index of multigenic resistance plants follows the same trend as the susceptible plants, but at a lower level. Thus, the susceptible allele is still in effect, but it is suppressed by the modifiers. In the monogenic resistant plants no symptoms whatever occur. Thus, when the susceptible allele is replaced by the resistant allele an entirely different process is introduced which is much more complete in its action and is not affected in any way by the modifiers. The same comparative responses of these genotypes in cabbage and in tomato occur when potassium is reduced to a lower level or nitrogen to a high level in the nutrient.

These are but a few of the many examples of the effects of environmental factors on expression of resistance. Such knowledge is important to the breeder in assaying his material. It is also desirable as a prelude to a study of the nature of resistance. During the past 50 years many cases have been studied in which substances fungicidal or fungistatic have been shown to occur within the host tissue. It is not proposed to go into these in detail here since they have been reviewed elsewhere (5, 48). Some general comments will be made. In a few cases such materials have been tied down as the basis of varietal resistance e.g., onion phenols (48), but for the most part they have not. This does not mean that they are not important, but it does mean that for the most part their toxicity is general and not specific and thus not clearly associated with high resistance and not correlated with sensitivity of pathogenic or nonpathogenic races of a pathogen. There is, however, a growing body of evidence that contact of the pathogen or non-pathogen with the host substrate may lead to metabolic changes in the latter which limit or prevent infec-

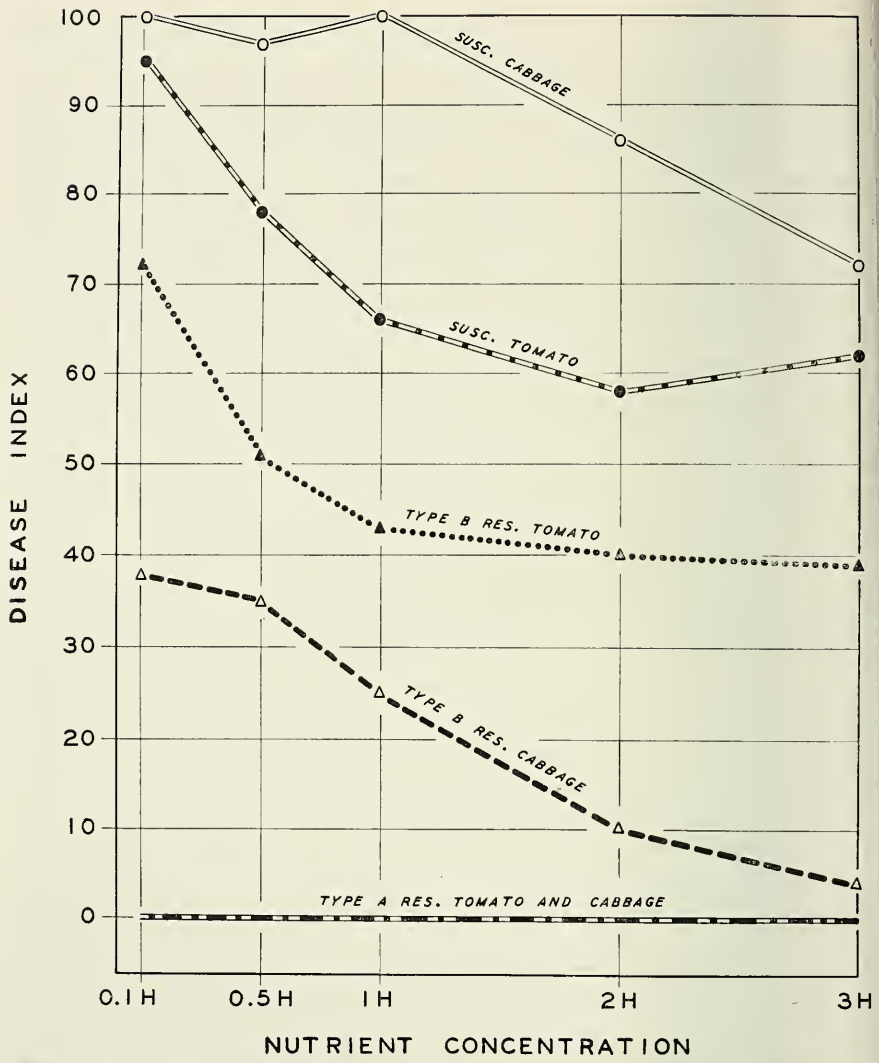

FIGURE 2. Development of cabbage yellows and tomato Fusarium wilt in susceptible, monogenic (Type A), and multigenic (Type B) resistant varieties fed with various concentrations of Hoagland solution.

tion. Just 50 years ago M. T. Cook and associates (13) suggested that, in surface wounds of plant tissue, polyphenoloxidases in the host tissue released phenols which protected the host from invasion. It has been shown by Muller (29), Kuc (25) and others that as inoculum is applied to certain plant tissue, inhibitive substances are produced which do essentially what Cook suggested. The nature of these materials

varies, but they are often phenols and/or quinones. It is to be emphasized, however, that they are not highly specific, and in my opinion are not to be taken as explanation of the types of resistance we have been talking about. This mechanism, however, is probably extremely important in protecting green plants generally from a host of miscellaneous microorganisms. It also might be theorized that our sophisticated pathogens of various green plants are those which by one mechanism or another get by this general defense barrier. When it comes to consideration of resistance to a specific pathogen or a pathogenic race thereof one must necessarily be concerned with the more specialized materials and mechanisms. One is tempted therefore to investigate cases where one or at least very few resistance genes are involved and where well-defined pathogenic races are concerned. It also has been learned by experience that we must be as much concerned with processes as with products, and in view of the potential effect of environment on the expression of resistance the more precise the control of the experimental environment the better. Furthermore, the relation of one process to another is probably just as important as any one process.

I can best illustrate what I mean by asking your indulgence while I refer to the old story of onion smudge resistance. It was first shown that colored bulbs were resistant because of colorless soluble phenolics in the dry outer scales. They are so closely associated with the color compounds in structure that they are considered to be part of the color metabolism. Geneticists (12) later showed that three gene pairs (and therefore three gene-controlled processes) are associated with color. The first of these (*Rr*) determines whether color is red (*RR* or *Rr*) or yellow (*rr*). For the expression of this process it is necessary to have the dominant gene *C* present in the heterozygous (*Cc*) or homozygous state (*CC*). If this epistatic gene is recessive, the bulb is white even though the potential for color and resistance is present. Moreover, even though *C* is present, the process controlled by a third gene pair *Ii* may still negate its effect. This process inhibits the effect of both *R* and *C*; if it is homozygous *II* the inhibition is complete, if it is heterozygous *Ii* inhibition is about half complete, both as to color and resistance. This is shown in Table 1. A large number of progenies homozygous *rr* and *CC*, but segregating for the *I* gene were exposed to the smudge organism and divided into three classes on the basis of color (23). The smudge index for the *Ii* class coincides very closely with the means of the indices for the *II* and *ii* classes, indicating that *I* and *i* have about equal influence on the enhance-

Table 1. Smudge Disease Indices in F_2 Segregating Progenies from Several Crosses Between Yellow (*rrCCii*) and White (*rrCCII*) Parental Lines

Color class	Genotype	Smudge index
White	*rrCCII*	66
Cream	*rrCCIi*	42
Yellow	*rrCCii*	13

ment or suppression of color and resistance where the other alleles are *rr* and *CC*. Before we know all about this case we should understand the nature of all three processes.

I should like now to give attention to a few studies which, although they do not give us the complete picture, do illustrate the present trend of approaches to the question of physiology of disease resistance. The hypersensitive reaction of highly resistant hosts to the invading pathogen, as is found commonly with rusts and powdery mildews, has been the subject of a number of elegant studies with classical histological and cytological techniques. These show a series of reactions between haustorium and host cell usually leading to a complete collapse of both. In recent studies by White and associates (27, 28, 37, 51) at Sydney, Australia, some interesting new light is thrown on this phenomenon. Using the powdery mildew fungus, *Erysiphe graminis* DC., and susceptible, semiresistant and highly resistant barleys they studied penetration and subsequent development by histological and biochemical methods. The number of haustoria per infection court and the subsequent development of external mycelium were in inverse ratio to the degree of resistance, i.e., 20 to 30 haustoria in susceptible, 10 to 15 in semiresistant, and 5 to 12 in highly resistant. However, respiration rose much more rapidly in resistant tissue than in susceptible tissue. This rise was apparently not confined to the invaded epidermal cells, but was pronounced in the non-invaded mesophyll cells. This indicated an unusual response of uninvaded resistant cells which was triggered by the relatively few haustoria in the infection court. Following the increase in respiration of the resistant mesophyll cells, collapse and necrosis occurred. From such tissue a substance was isolated which was shown to increase respiration in leaf tissue apparently by uncoupling phosphorylation which normally limits the rate of respiration. It is emphasized that this occurs in the uninvaded mesophyll cells, and thus appears to be a host reaction and not a fungus reaction. Following necrosis around the invaded cell a phenolic substance appears which diffuses into the invaded cell and poisons

the haustoria. The most important part of the resistant reaction appears to be the ability of resistant mesophyll to react to some sort of a stimulus from the invaded cell in such a way that a vivotoxin is produced, while no such reaction occurs in the susceptible. This is quite in contrast to the situation in onion where phenolic compounds are released in the outer scales without any stimulus from the pathogen, and when the latter is applied to the living epidermal cell of a resistant bulb there is no resistant reaction.

The same phenolic substance was found in healthy susceptible and semi-resistant barley plants, indicating that it is not unique in the highly resistant one. What is unique in the latter is the quick response to the stimulus from the invaded cell. This response is undoubtedly dependent on a specific enzyme system. If such is the case, it is not so difficult to visualize why the same or similar cells react differently to different pathogenic races, i.e., why one variety and even the same cell is susceptible to one race and highly resistant to another. The difference between races is probably a difference in the trigger they produce directly or in partnership with the invaded epidermal cell. The phenolic or other toxin produced after necrosis may well be the same regardless of the pathogenic race. The difference in resistance probably lies in the difference in the trigger and in the respiratory pattern.

Uritani and associates (42, 43) have carried on an extensive study of resistance in sweet potato fleshy roots to black rot (*Ceratocystis fimbriata* Ellis and Halsted). This disease is a slowly progressing dry rot. There is a definite reaction of noninvaded tissue adjacent to the infected area. This includes increase in respiration and accumulation of polyphenols and coumarins. There is also accumulation of ipomeamaron, a sesquiterpene, in the infected tissue. However, these same responses can be induced by poisonous chemicals, such as mercuric chloride, when applied to the tissue. In the uninvaded area near infected tissue antigens were present which were not found in normal tissue. One of these antigenic components was shown to be a peroxidase. Another, component B, has not been characterized. Some varieties of sweet potato are more resistant than others to the rot of the fleshy root. In general, respiration and the amounts of these new protein components are higher in the uninvaded marginal tissue of resistant fleshy roots than in that of susceptible roots. It is suggested that resistance may be concerned with a change in protein synthesis induced by the pathogen through its own metabolites or those of the infected host cell which diffuse into and alter the metabolism of uninvaded cells.

A similar approach has been made by Tomiyama and associates (41) to the nature of resistance in potato to the late blight organism (*Phytophthora infestans* [Mont.] DeBy.). Here we have two genetic types of resistance, as in cabbage yellows, one being monogenic, the other multigenic. There are also distinct pathogenic races of the fungus and within such races differences in virulence. I shall confine my remarks principally to monogenic resistance in the host, and primarily to the reaction of resistant and susceptible tubers. After penetration there is a rapid hypersensitive reaction of the tissue characterized by necrosis, whereas in susceptible tissue this reaction is absent or delayed. In the resistant tissue there is a greater increase in respiration and in polyphenol oxidase activity following invasion than in susceptible tissue. These processes result in the accumulation of phenols in the necrotic tissue, indicating that the increase in amount and probably in type of oxidase activity results in the breakdown of compounds containing phenols, releasing them where they might act as fungistatic or fungicidal agents. If they become oxidized to quinones they become more potent in this regard, combine with proteins, and polymerize to form melanins. Their function in resistance is most likely to be in the first of these three stages and not when necrosis becomes visible due to the last stage. That this series of events is initiated by a stimulus passed on from the invaded cell to underlying uninvaded cells is neatly shown by the ingenious experiment of comparing reactions of tuber slices of differing thickness when inoculated with a race to which the tuber is resistant. If the slice of resistant tissue is too thin a susceptible reaction occurs; if the slice is a little thicker a resistant reaction occurs. Tomiyama concluded that a minimum number of cells beneath the invaded cell was necessary to bring about enough metabolic activity around that cell to confine the pathogen. It should be borne in mind that variety A, resistant to race 1 and susceptible to race 2, reacts in the above described manner only to race 1 and not to race 2, while in variety B, susceptible to race 1 and resistant to race 2, just the opposite occurs. It may well be assumed that the host cells of the two varieties are much alike as are the phenolic compounds which build up in the necrotic tissue. They are both capable of reacting as resistant or as susceptible cells. What appears to make the difference is the potential of the invading fungus. Race 1 has the property of stimulating the resistant reaction in variety A, but not in variety B, while race 2 does this in variety B and not in variety A. The wild race 0, however, is capable of stimulating the resistant reaction in A and B. It is evident then that the resistant

character is a latent process which is triggered by the pathogen and only when the trigger is carried by the pathogenic race concerned. If it is not there, the host is susceptible. Unfortunately the fungus loses a trigger apparently by a single-step mutation and thus a "new" race appears and a resistant variety falls down (18). This situation, as we see it in late blight, again emphasizes that we need to give attention to processes as well as products in resistant host metabolism.

Anderson and I (3) have studied recently the reaction of resistant Charleston Gray and susceptible Dixie Queen and Peacock watermelons to the anthracnose organism (*Colletotrichum orbiculare* [Berk. and Mont.] V. Arx). Charleston Gray is highly resistant to race 1, all three are susceptible to race 2. When race 1 penetrates the susceptible leaf the hypha invades rapidly, and cells die rapidly. When the same race penetrates the resistant leaf it seldom invades more than one or two cells, which die, but there is no necrosis of the underlying cells as in the cases of potato blight and barley mildew. On the contrary the underlying tissue responds by relatively rapid cell division, which proceeds to a certain point and stops (Figure 3). Whether this proliferation has any place in the resistance mechanism is not clear, but there is indirect evidence that it has. A similar reaction occurs in resistant fruit. However, if an inoculated piece of the fruit is removed to a glucose substrate, the tissue does not so react and the lesion develops as on a susceptible fruit. If race 2 is applied to the resistant fruit, no proliferation occurs and a normal lesion develops. It appears that the resistant reaction depends on the support of the entire fruit or plant while in the potato blight case only a small number of cell layers is required. Phenols are conspicuously absent in the resistant reaction in watermelon. All races of the pathogen so far described, except race 2, appear to trigger the resistant reaction.

The nature of resistance to Fusarium wilts has intrigued us for a long time. We have confined our attention chiefly to monogenic resistance in tomato and cabbage. Here there is meager invasion of roots of resistant plants, and thus the resistant reaction, whatever it is, is functional. It can be shown to occur throughout the plants, however, by introducing bud cells of the pathogen in the vascular system of stem cuttings, rooting the latter, and exposing plants to favorable temperature for wilt (35). With tomato there is a temporary wilt symptom in resistant plants at the same time it appears in comparable susceptible plants. At this time the live organism can be isolated throughout resistant and susceptible plants. Continued sampling

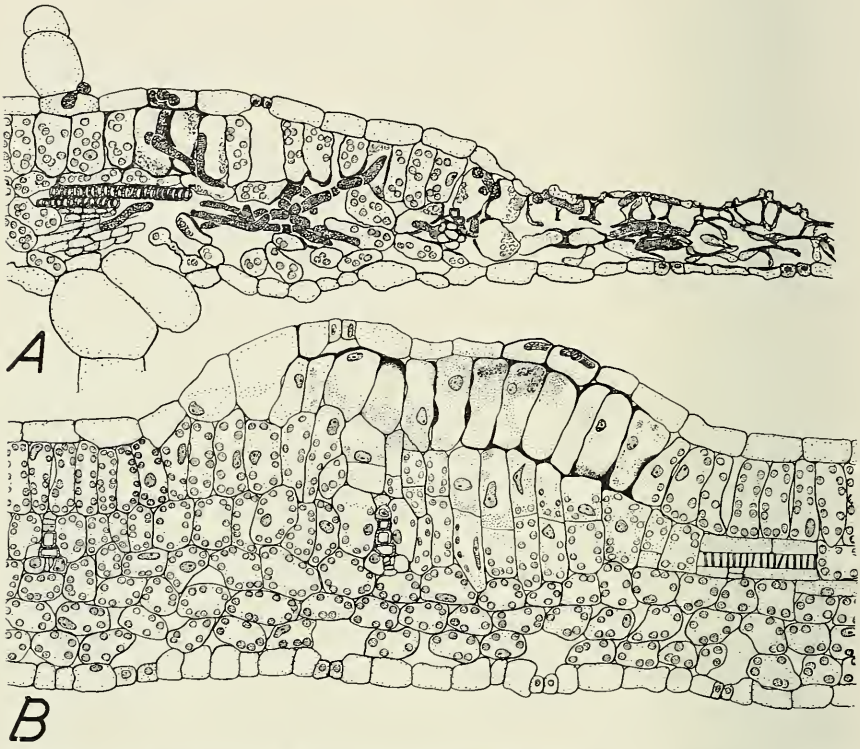

FIGURE 3. Comparison of reaction of a susceptible (A) and a resistant (B) variety of watermelon to invasion by the anthracnose organism.

of the resistant plants shows a gradual decline of the live organism in the symptomless resistant plant, disappearing first in leaflets, next in petioles, and finally after 35 days it was reisolated only from the lower stem. This gives the outward picture that the organism is starving in the resistant plant. What happens is shown in a cross section of a resistant stem 25 days after inoculation (Figure 4). Dead mycelium is seen in the oldest vessels. The necrotic area is surrounded by fungus-free secondary xylem. This picure looks superficially much like that observed at the surface with examples cited above. Is the fungus starved or poisoned after an initial start? Crude extracts of resistant and susceptible tissue show no differential effect on growth of the organism. The same is true when xylem exudate is used. The fungus probably depends to a considerable extent on its excretion of depolymerase to break down pectic materials in the tracheal walls to provide energy for growth and continued activity. Deese and Stahmann

(15) sterilized stems of resistant and susceptible tomatoes by propylene oxide and used them as substrates for the fungus. Again there was little difference in superficial appearance of growth on the two substrates. However, when the substrates were examined a few days after inoculation the residual depolymerase in the susceptible substrate was considerably greater than that in the resistant substrate. When juice from inoculated resistant and susceptible plants was examined for depolymerase there was a decided increase in the latter over that in the former (14). These facts indicate either that the resistant tissue is a poorer substrate for production of the adaptive enzyme or that the enzyme is inactivated more rapidly as it is produced in the resistant substrate. The latter explanation seems more plausible in view of apparent equal growth on the two substrates. In any case, this differential factor may have some effect on resistance, but it falls short of explaining the wide difference between the reaction of susceptible and monogenic resistant plants to the wilt organism.

Evidence that resistance is bound up more closely with host metabolism was furnished first by Scheffer and myself (35). When cuttings were inoculated as described above and a number of different alcohols were added in minute amounts to the liquid in which cuttings

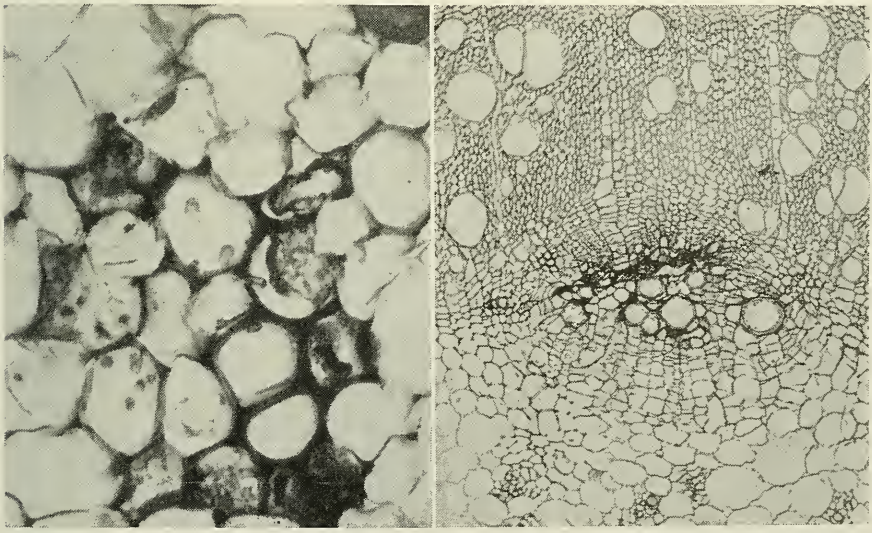

FIGURE 4. Relation of the Fusarium wilt organism to the lower stem tissue of susceptible and resistant varieties of tomato. While plugging and browning proceeds in the susceptible variety with little or no host reaction, the fungus becomes confined and finally dies in the xylem of the resistant host. Secondary xylem rapidly surrounds the infection pocket.

were immersed, it was found that 0.2 per cent ethyl alcohol so altered the metabolism of the resistant plant that normal wilt symptoms developed and the resistant plant had, in effect, been converted to a susceptible one. This was repeated many times. It was evident that the alcohol had retarded or stimulated some phase of the host metabolism, and in so doing, had effectively blocked the resistance mechanism.

Further evidence that resistance was bound up with host metabolism was shown by Gothoskar, Scheffer, Stahmann, and myself (20) when a number of respiratory inhibitors were used instead of alcohol. 2,4-Dinitrophenol, thiourea, sodium fluoride, and sodium diethyl dithiocarbamate each broke resistance in a manner similar to ethyl alcohol (Figure 5). We have no evidence here that the pathogen stimulates a resistant mechanism as is suggested in some of the examples cited above, although this possibility needs further investigation. It is clear, however, that the resistance process is bound up with host metabolism and that substances which inhibit phosphorylation or certain other steps in the respiratory system block that mechanism. In a parallel study with cabbage yellows, Heitefuss, Stahmann, and Walker (22) introduced bud cells into resistant and susceptible cuttings and measured the oxidative enzyme activity with an ascorbic acid substrate. The oxygen consumption rose gradually as symptoms developed in the susceptible cuttings. Despite the absence of symptoms in the resistant cuttings it rose abruptly to a much higher level and then declined sharply. This indicated an abrupt response in metabolism in the resistant host which is probably connected in some way with the resistance mechanism.

Resistance to cucumber mosaic virus is monogenic in spinach (32), cowpea (38), and cucumber (49). The virus-host relationship, however, differs in the three diseases. In spinach there is little or no multiplication of the virus and no symptoms at 16° and 20°, while at 28° there is systemic invasion and nearly as rapid killing as in plants of the susceptible variety. In cowpea, resistant plants show a local lesion reaction of inoculated leaves and no further virus multiplication, while in susceptible plants systemic mottle and stunting occurs. In cucumber the virus multiplies systemically in susceptible and resistant plants, but the latter outgrow symptoms promptly at moderately high temperature. After the initial stages, the virus titre in resistant plants tends to decline although in both susceptible and resistant plants virus titre assumes a cyclic pattern (50). Menke and I (25a) have shown that there is a greater increase in respiration in inoculated resistant cowpea leaves than in susceptible leaves, but the rate de-

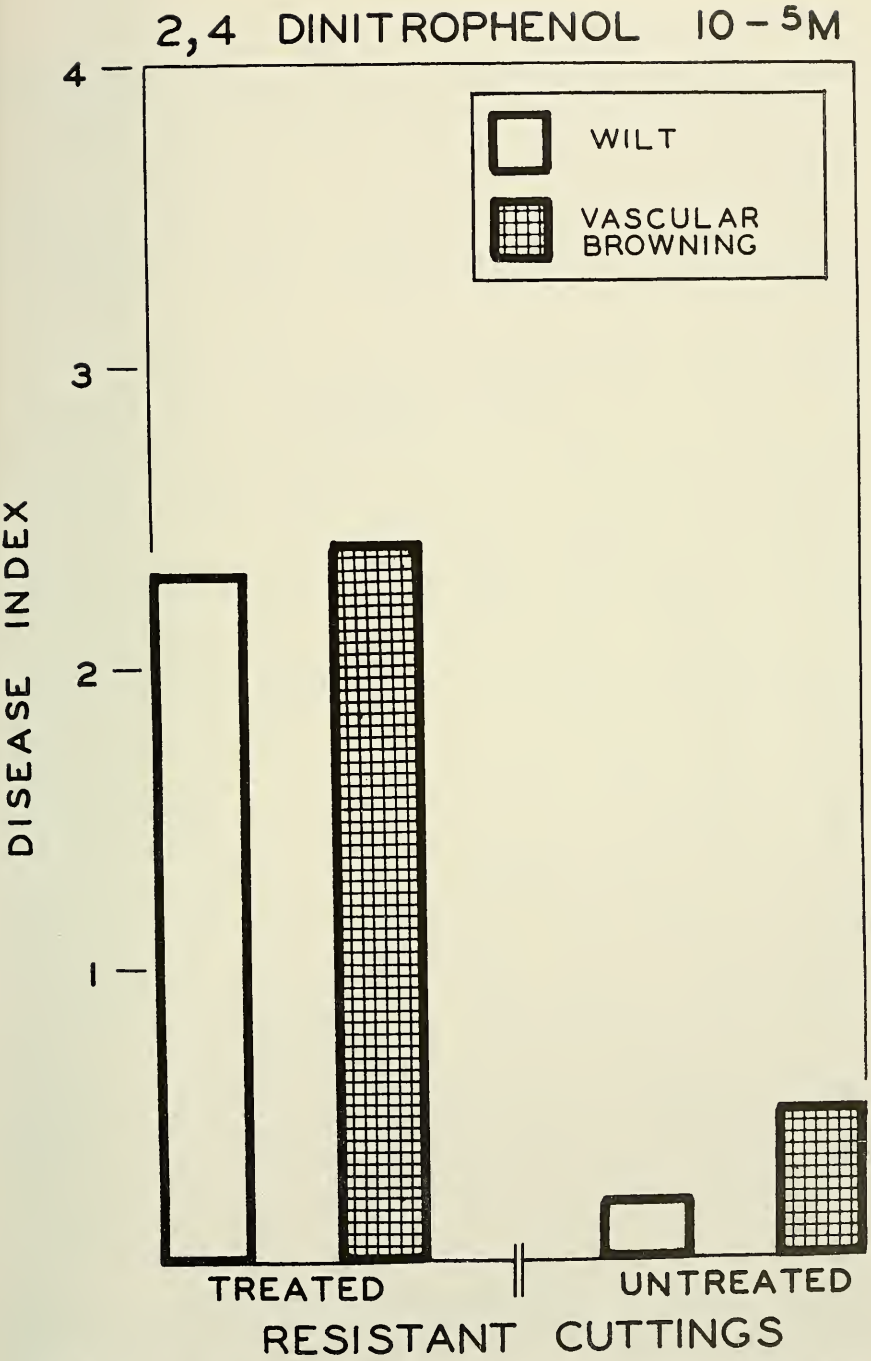

FIGURE 5. The effect of dinitrophenol on the wilt and browning indices of inoculated cuttings compared with inoculated untreated cuttings of a resistant variety of tomato.

clines in the former at about the time local lesions occur. This is not unlike a common reaction of hypersensitive reaction to fungus pathogens, but in this case there is no evidence that polyphenol oxidase is involved.

In the case of cucumber, where systemic infection occurs in both resistant and susceptible plants, a different situation prevails. Closely similar horticultural varieties which differed chiefly in resistance or susceptibility to mosaic were inoculated by rubbing cotyledons of plants in which the first leaf was about one-fourth normal size, and the third leaf was barely visible. Plants were observed for about 14 days in a fairly constant temperature of 20° and a 12-hour day period with constant light of about 1,000 foot-candles. Symptoms were least pronounced in the first leaf and most pronounced in the third leaf of susceptible plants at the end of the period. They were in each case visible, but less pronounced in the resistant plants. Virus assays made throughout the period showed lowest virus titre in the first leaf and highest in the second leaf. While the titre varied in each leaf from day to day it was usually somewhat lower in the resistant than in the corresponding susceptible leaf. In each leaf the uptake of O_2 was usually greater at any given interval in the inoculated leaf than in the uninoculated leaf. The difference was relatively small in the first leaf, greater in the second leaf, and greatest in the third leaf. The increase in ratio of O_2 uptake, therefore, was correlated more closely with symptoms than with virus titre. The ratio was also smaller in the resistant when compared with the corresponding susceptible leaf. Of several oxidase enzyme systems examined the peroxidase activity was the only one which showed a marked effect of infection and a wide difference between resistant and susceptible leaves. In susceptible leaves peroxidase rose most rapidly in the third leaf and least in the first leaf. It also rose more rapidly in susceptible than in corresponding resistant leaves. The change in peroxidase did not always follow that of O_2 uptake nor that of virus titre (Figure 6). It is to be noted that while in some of the cases of hypersensitive reaction cited above there is a tendency for a rapid increase in respiration of the resistant host and in some cases of a similar increase in oxidative enzymes (e.g., cabbage yellows), quite the opposite relation occurs here. The mechanism of resistance to mosaic in cucumber, although monogenic, is one which permits the multiplication of the virus, but at the same time manages to restrict its detrimental effect so that growth and fruit production of the host assumes a rate close to that of the uninoculated control. Whatever the resistance mechanism turns out to be, it is

obviously one which holds respiration and peroxidase activity closer to normal than in the susceptible plant.

The last group of diseases I wish to discuss briefly are those in which a powerful toxin is produced by the pathogen which diffuses well in advance of the latter and which can be produced *in vitro* and purified. This offers the investigator an opportunity to study resistant and susceptible reaction without interference of the organism. In study-

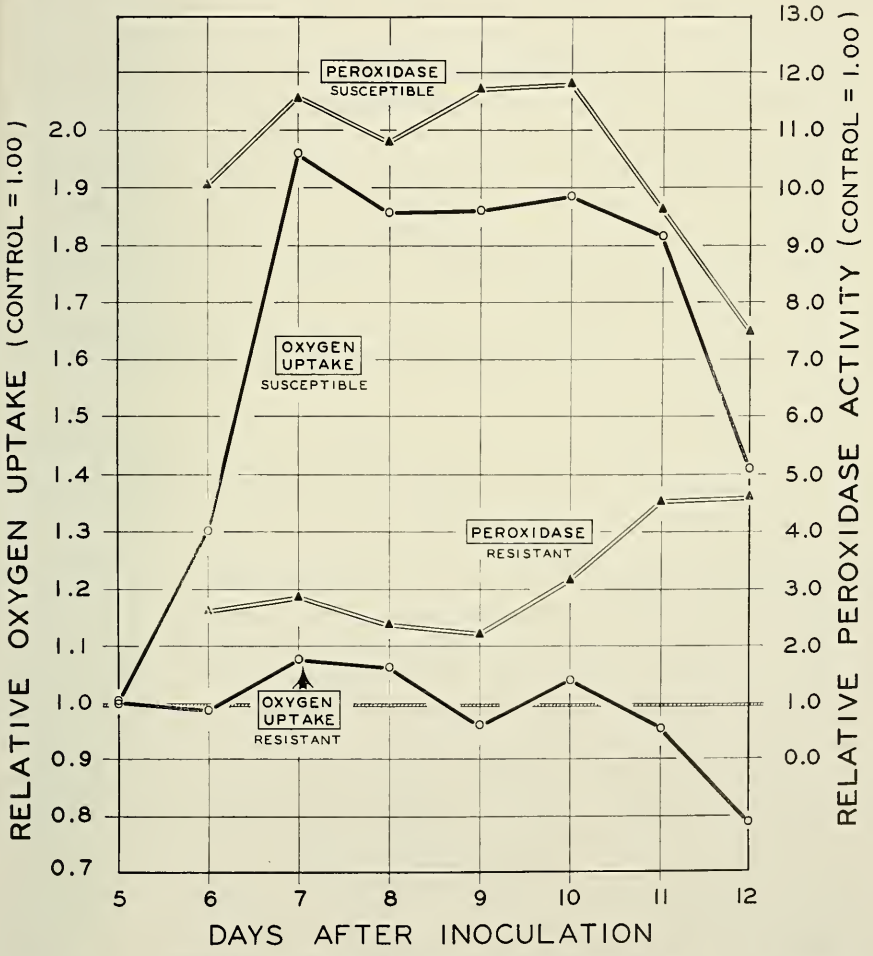

FIGURE 6. Comparison of the oxygen uptake and peroxidase activity of tissue of the third leaves of susceptible and resistant cucumber varieties inoculated with cucumber mosaic virus by rubbing the cotyledons with infectious juice from a diseased plant.

ing respiration and enzyme pathways in diseased tissue it is always difficult to distinguish between activity of the parasite and that of the host. Victoria blight of oat (*Helminthosporium victoriae* Meehan and Murphy) has now received considerable study with regard to the nature of resistance. The toxin, victorin, which is probably a polypeptide, when prepared in highly purified form produces the same symptoms on susceptible oats as does the pathogen itself (33). Resistance is of a high order and is controlled by a single recessive gene. The physiological studies of resistance by Romanko (34) are of special interest. When cuttings of resistant and susceptible oats were exposed to the toxin the latter was readily demonstrated in the leaf tissue of susceptible plants by bioassay. Little or none was demonstrated in resistant tissue. Susceptible tissue responded to the toxin by pronounced increase in respiration as indicated by O_2 consumption, while in the resistant tissue the change in respiration was not significant. It would appear that the resistant tissue is capable of metabolizing the toxin without any detrimental effect. Presumably the fungus is unable to infect unless the exotoxin first kills host cells in advance. A similar case has been described recently by Scheffer and Pringle (36) for the highly specific exotoxin of *Periconia circinata* (Mangin) Sacc., which incites the milo disease of sorghum.

In the case of tobacco wild fire, the organism (*Pseudomonas tabaci* [Wolf and Foster] Stevens) produces another exotoxin in quantity in culture medium. This also in purified form produces the same symptoms as the organism. Unlike victorin, the wild fire toxin is not specific since it produces characteristic chlorosis on a wide range of plants. Highly resistant varieties of tobacco have been produced. Unlike the case in oats, the wild fire exotoxin will produce symptoms on a highly resistant variety. When the resistant variety is inoculated, however, symptoms do not appear or, if they do, they are very slight. Garber (19) confirmed earlier reports (16) that bacteria multiplied more rapidly in infection sites of susceptible leaves than in comparable resistant leaves and showed a higher buffer index in juice of susceptible as compared to resistant leaves. He suggests that the low buffer capacity of resistant tissue allows a rise in alkalinity in the infection site which is detrimental to growth of the pathogen and also promotes inactivation of the exotoxin.

SUMMARY

In this discussion I have tried to depict some of the important advances in our concepts of disease resistance during the past 50

years. The existence of definite genes controlling resistance has been demonstrated in hundreds of instances, and they have been put to use for the benefit of mankind in hundreds of resistant varieties.

As in other plant characters the expression of disease resistance may be influenced by environal and nutritional factors. This response is most pronounced when the character is multigenic, and usually, but not always, least pronounced when the character is monogenic. A few cases have been described in which specific preformed fungitoxic materials in the host function as the main resistant character. There are instances known, on the other hand, where materials highly toxic to the pathogen are present in the host, but which appear to make no contribution to the resistant character. There is a growing body of evidence that resistance in many cases is bound up with a reaction of the resistant host to penetration. This may be manifested in the invaded cell, but is more often found in the form of disturbed metabolism of adjacent uninvaded cells. In such cells there is usually found an increase in respiration and sometimes of a specific enzyme. That the resistant character is intimately associated with one or another enzyme pathway is shown by the fact that resistance may in some cases be altered or broken down completely by enzyme inhibitors. Since the same host cells may be highly resistant to one race and completely susceptible to another race of the same pathogen, the resistant character is apparently dependent upon the balance and interaction of enzyme systems. The activation of such a substrate may well be triggered by the invading pathogen and the type of reaction, resistant or susceptible, may well be determined by a characteristic of the pathogen. In further elucidation of the nature of disease resistance of plants the investigation of the comparative physiology of susceptible and resistant plants and of the races of pathogen will be the major line of attack. This requires new men, new weapons, new points of view, and alert use of methods evolving rapidly in plant physiology and enzymology.

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Disease and Fungus Physiology

A symposium

sponsored by the Potomac Division

of the

American Phytopathological Society

at

its meeting at West Virginia University

Morgantown, West Virginia

April 24 and 25, 1962

INTRODUCTORY REMARKS

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PLANT PATHOLOGY is often contrasted with animal and human pathology in that we, in plant pathology, must of necessity deal with diseases as they affect large populations of plants, whereas the animal pathologists devote more of their time and effort to the study of diseases as they affect the individual. Although plant pathologists have long been interested in the mechanisms of disease production and the nature of parasitism, we have been handicapped by a lack of knowledge and understanding of the chemical nature of the processes involved. However, with the rapid advances now being made in biochemistry as applied to cellular and molecular biology, it is only natural that plant pathologists should be stimulated to turn their attention more and more to those abnormal physiological processes that take place in the cells and tissues of the individual diseased plant.

This problem is still further complicated by the fact that when we deal with the physiology of diseases caused by parasitic fungi we are concerned with the physiology of the pathogen, the physiology of the affected plant, and the reaction of the two. This symposium is a manifestation of the growing interest in the biochemical nature of those physiological processes that we call parasitism and disease. It has been designed to review some of the things that we have learned about the physiology of fungi as such, and to consider what we know about two types of fungus parasitism, namely mycoparasitism and the parasitism of the wilt-producing fungi. Any increase in our knowledge of these phenomena should help us to better understand the nature of the disease with which we as plant pathologists must deal.

Inasmuch as this symposium is part of the celebration of the 50th Anniversary of the Department of Plant Pathology of West Virginia University it is appropriate that we review briefly the history of research on fungus physiology in this department. I have asked for the privilege of doing this because most of my personal research has not been in this field and I should be able to treat the subject in a more or less objective manner.

Intensive work on the physiology of the fungi at West Virginia University began almost forty years ago with the appointment of Dr. L. H. Leonian as Assistant Plant Pathologist. Dr. Leonian had earn-

ed his Ph.D. at the University of Michigan under the direction of Dr. C. H. Kauffman, who in turn, had been a disciple of George Klebs. From Dr. Kauffman, he derived a consuming interest in the variability of fungi and the physiological basis of such variability. He was a firm proponent of the importance of physiological characters in the classification and identification of fungi, and believed that too much emphasis was placed on morphology. He was not interested in mycological herbaria and the study of dead specimens. This philosophy was strengthened by his extensive studies on the variability of *Fusarium moniliforme* and by his studies on the physiology and classification within the genus *Phytophthora.* His indictment of the traditional emphasis on morphological characters in species determination was on one occasion worded as follows: "We have been in the habit of describing the species according to its morphological characters; we have usually failed to make intensive cultural studies, and have endeavored to formulate fundamental truths by superficial observations. To many of us, form and size of spores and reproductive bodies constitute the sum total of the mycological concept, and the vital processes of fungi have little taxonomic value in our scheme of classification. Many of us, despite our professed liberalism, still cling to the notion that a species is as it appears to us at a given time. While any organism is a unit in itself, it may, nevertheless, exhibit only one or just a few of its component phases at a time."

It was Dr. Leonian's lament, and often stated conviction, that most worthwhile contributions to the science of plant pathology would come from a better understanding of the physiology of the pathogen and the physiological reaction between the pathogen and its suscept.

Dr. Leonian soon realized that effective progress in the study of fungus physiology would eventually lead to problems in chemistry. Not being trained as a chemist, Dr. Leonian sought the cooperation of University chemists but found that they were either preoccupied with their own problems or too heavily loaded with teaching duties to provide the needed assistance.

In his characteristic directness he turned to the obvious alternative of hiring a chemist for the specific purpose of assisting in the physiology of the fungi. Despite this administratively unorthodox approach to the problem, Dr. V. G. Lilly, a recent Ph.D. in chemistry but with little training in biology, was in 1934 appointed as "Physiologist" and assigned to the research on fungus physiology in cooperation with Dr. Leonian. Dr. Lilly has been active in the program since that date and over the years has not only become a well-qualified biologist but

has also helped many budding biologists to have a better appreciation of the chemistry and physiology of living organisms.

In 1945 the program suffered a great loss in the untimely death of Dr. Leonian. It then became evident that the continued progress in this research at West Virginia University would largely depend upon the person appointed to succeed Dr. Leonian. There were those who, in harmony with the current philosophy, believed that the department would be served best by a person with more interest in the applied fields of plant pathology, whose work could show more economic value to the farmer in terms of dollars and cents. There also were some who believed that the best interest of the department would be served by a taxonomic mycologist. But fortunately for the program, Dr. H. L. Barnett was appointed with the definite and specific understanding that he would devote his efforts to the continuation of the program initiated and so ably carried forward by Dr. Leonian. As to whether Dr. Barnett has done this, the record speaks for itself. For my part I have never known two people with diverse backgrounds who have worked so closely and so harmoniously as a team and with such productivity as the team of Lilly and Barnett, a team of which we are justly proud on this our fiftieth anniversary.

The Relation of Fungus Physiology to Physiology of Disease

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DISEASE PHYSIOLOGY and fungus physiology are related as a building is related to its foundation. Plant pathologists spend much of their efforts in combatting the activities of fungus pathogens. To do this effectively, it is necessary to know where the pathogen is strong and where it is weak. Much of this information can be learned by studying the pathogen in the laboratory. It should then be easier to study the more complex situation of disease in the greenhouse and in the field.

In this department, fungi have been studied for 50 years as pathogens and for the past 40 years they have been studied intensively as fungi. Each area has supported the other. This section of the symposium will deal with the effects of nutrition and environment upon selected fungi, not all of them pathogens.

Problems like the following arise when a plant pathogenic fungus, or for that matter any fungus, is cultivated and studied in the laboratory. What medium should be used, natural, synthetic, semi synthetic, liquid, or agar? What carbon and nitrogen sources should be used, and at what concentration? Should vitamins be added? Will the pH of the medium be critical, and how can it be maintained at desired levels? Should media be sterilized by autoclaving, by filtration, or by the use of a fumigant such as propylene oxide? How can maximum sporulation be obtained, or how can certain species and isolates be induced to sporulate? How should growth and sporulation be measured? Should the cultures be grown in light, darkness, or alternating light and darkness? What temperature should be used? How much variation exists among isolates of the same species? All of these questions, and many more, should be considered in the light of another question: What is the purpose of the study?

Fielding in "Joseph Andrews" remarks "It is a trite but true observation that examples work more forcibly on the mind than precepts." We will turn now to a consideration of some particular fungi as examples.

The medium. The composition of the substrate is of the utmost importance in culturing fungi. This is as true for fungi in nature as in the laboratory. Fungi must obtain from the substrate all of the elements and compounds they require to support their life processes. A pathogen growing in its host obtains from the host all of the metabolites it requires. One medium can differ from another in only two ways: the compounds present, and the concentration of each. This is just as true of the host as of laboratory media. It is the composition of a medium that is important, not its name. How many of you recall the composition of Richard's medium?

Is there any universal medium suitable for the cultivation of all plant pathogenic fungi? Is potato-dextrose-agar medium the answer? The answer to both of these questions is "no." Only a minimum number of questions can be answered by the use of one medium, even PDA.

Carbon compounds utilized. Apparently, every naturally occurring organic compound is utilized by one or more microorganisms. Fungi are versatile in this respect; Dobson (1962) found a number of species to grow well in kerosene as a carbon source. A simple and common type of experiment is to determine which sugars are utilized by a fungus. It is frequently observed that a trace of growth occurs on all the sugars tested. This is particularly true when an amino acid such as asparagine (or a mixture of amino acids such as casein hydrolysate) is used as a nitrogen source. The fact may be overlooked that many fungi utilize amino acid-carbon. Lilly and Barnett (1956) showed that nine species of fungi would utilize asparagine as a source of carbon and nitrogen. In such experiments, a control medium without a carbohydrate source should always be included among the media.

Fusarium lycopersici grows as rapidly on casein hydrolysate as on glucose-carbon (Lilly and Barnett, 1956a). Thirty-two out of 34 species grew on a casein hydrolysate medium without added carbohydrate, whereas *Phycomyces blakesleeanus* and *Schizophyllum commune* made only a trace of growth unless glucose was added to the casein hydrolysate medium. Thus, an amino acid or mixture of amino acids may be a good source of nitrogen, but the amino acid-carbon may be unavailable to certain fungi.

In nature fungi rarely, if ever, come in contact with only a single sugar. Is it safe to conclude that experiments with individual sugars will reveal the spectrum of sugar utilization? Is the availability of a given sugar modified by the presence of other sugars?

Waters, Lilly, and Barnett (1953) found that *Sordaria fimicola* made only a trace of growth on a sucrose-asparagine medium; less than

10 mg of mycelium/culture was produced in 124 days. However, when fructose, galactose, glucose, or L-arabinose was added to the sucrose media more mycelium was produced than on monosaccharides alone (Figures 1, 2, 3).

Lilly and Barnett (1956) compared the rate and amount of growth of 49 species of fungi on asparagine media containing D-arabinose, L-arabinose, and DL-arabinose. With one exception, *Sporobolomyces salmonicolor*, L-arabinose was utilized more readily than D-arabinose. Many species grew about as readily on DL-arabinose as on L-arabinose, indicating that the presence of the more readily utilizable isomer increased the rate of utilization of the other isomer. Figure 4 shows the rate and amount of growth of *Neocosmopara vasinfecta* on D-and L-arabinose separately and on mixtures of D- and L-arabinose. Other experiments have shown that the rate of growth of *Chaetomium globosum* on L-arabinose was increased by the presence of glucose (Figure 5).

L-sorbose occurs but rarely in nature and is a poor carbon source for many fungi. In admixture with other sugars, L-sorbose may decrease or increase growth (Lilly and Barnett, 1953). The inhibition due to

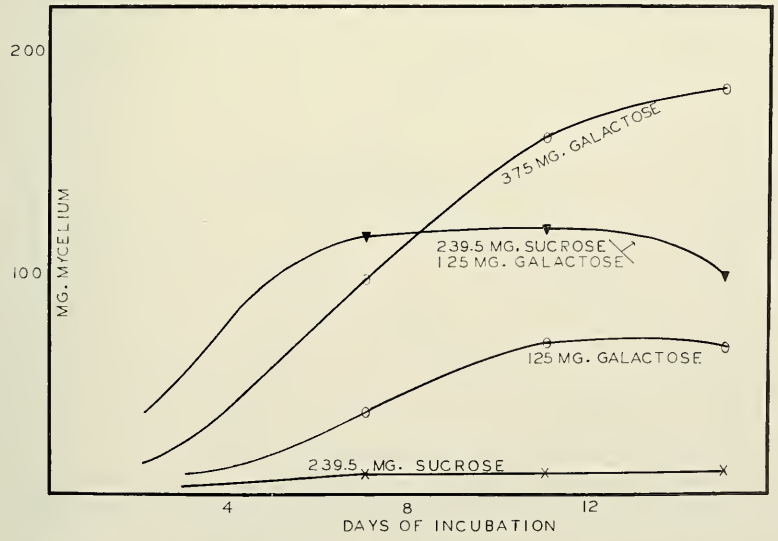

FIGURE 1. Growth of Sordaria fimicola on media containing sucrose, galactose, and a mixture of galactose and sucrose. Note that more mycelium was produced on the mixture of galactose and sucrose than on either of these sugars separately.

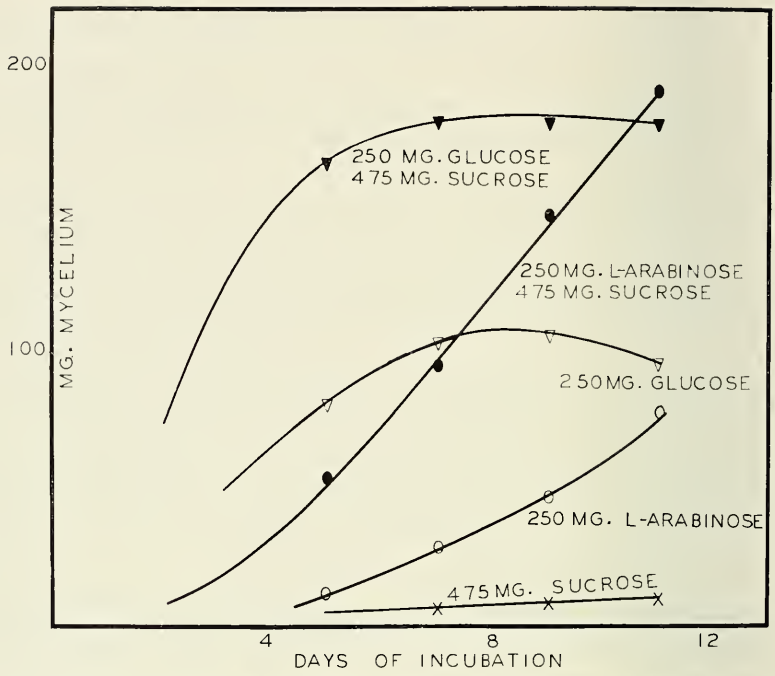

FIGURE 2. Growth of Sordaria fimicola on mixtures of glucose and sucrose, and L-arabinose and sucrose. Note the increased growth on the mixtures compared with the controls.

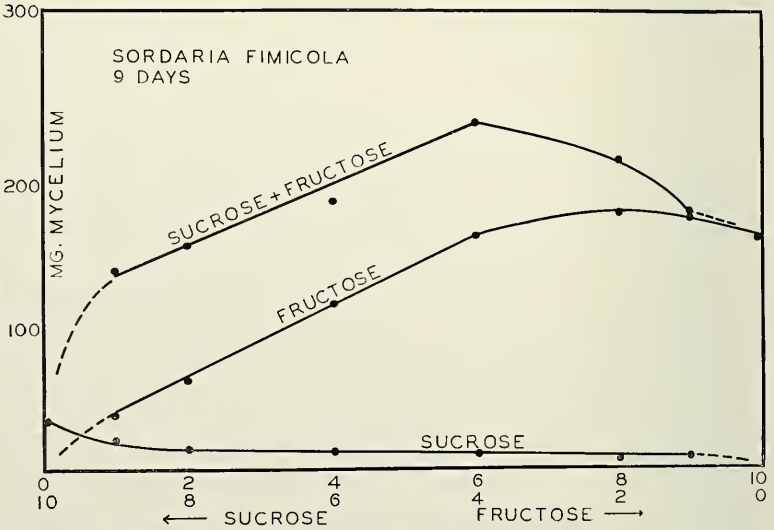

FIGURE 3. Growth of Sordaria fimicola on media containing varying amounts of sucrose and fructose. Note the increased growth on the media containing sucrose and fructose compared with the media containing fructose alone.

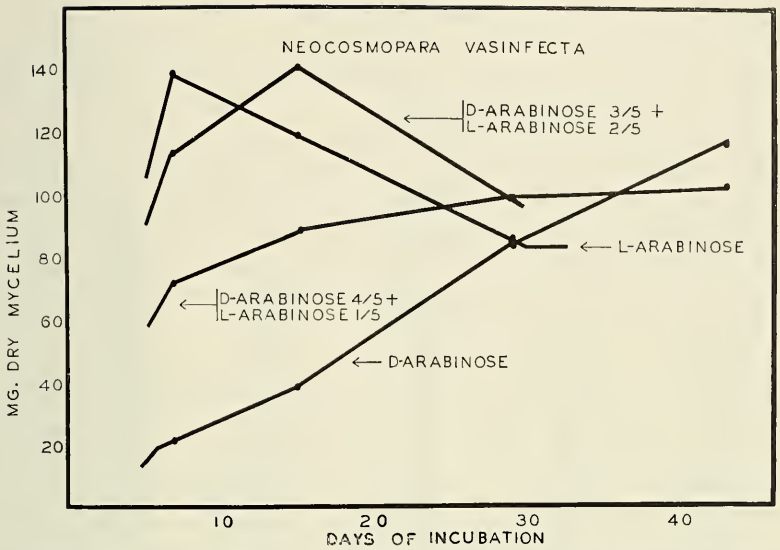

FIGURE 4. Rate and amount of growth of Neocosmospora vasinfecta in media containing D- and L-arabinose separately, and in mixtures of these sugars.

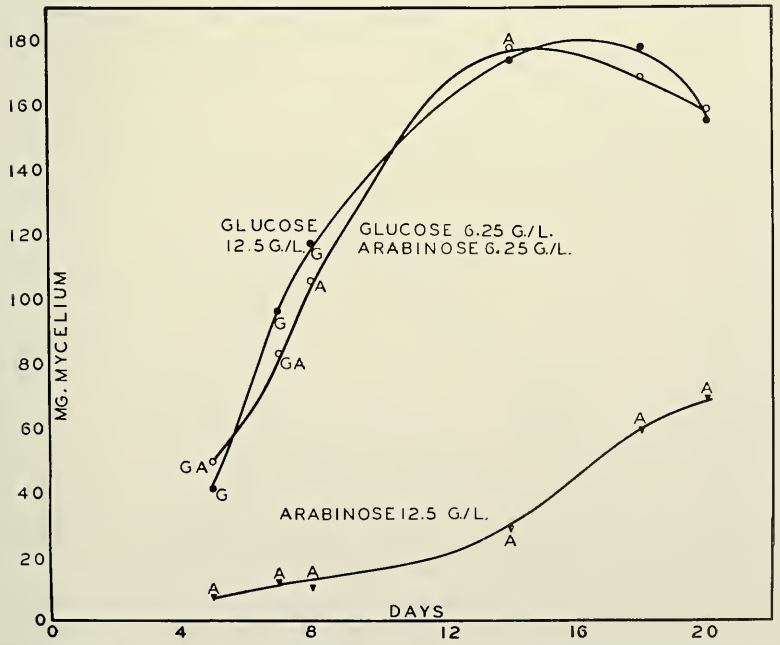

FIGURE 5. The effect of D-glucose on the utilization of L-arabinose by Chaetomium globosum. G and A designate glucose and arabinose, respectively, in the filtrates.

L-sorbose is dependent upon the sugar used and the temperature of incubation, as shown in Figure 6.

Brown (1958) grew *Chaetomium globosum* in an asparagine medium which contained 20 g/l of L-sorbose and also in aliquots of the same basal medium to which 20 g/l of D-fructose, D-galactose, L-arabinose, maltose, and sucrose were added. Very little growth resulted. In a parallel experiment, the concentrations of the above sugars was reduced to 15 g/l; 5 g/l of D-glucose was added to all media. Some of the results are shown in Figure 7.

The simple examples discussed above are far from exhausting the variables, which might, and do, influence the utilization of sugars by fungi. *Chaetomium globosum* was cultured on media containing different nitrogen sources and on maltose alone, and on mixtures of maltose and L-sorbose (Figure 8). Note that L-sorbose inhibition was slight when Casamino Acids were used, moderate when L-arginine was used, and almost complete inhibition occurred when L-asparagine was used (Lilly and Barnett, 1953).

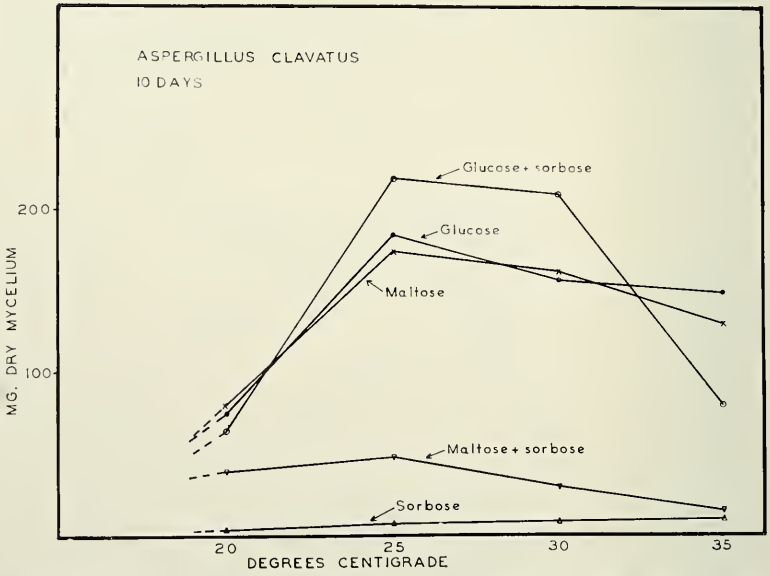

FIGURE 6. Growth of Aspergillus clavatus (10 days) on sorbose, glucose, and maltose alone and on mixtures of glucose and sorbose, and maltose and sorbose at 20, 25, 30, and 35° C. Note that sorbose did not inhibit growth on the glucose medium except at 35° C, whereas sorbose inhibited growth on the maltose medium at all four temperatures.

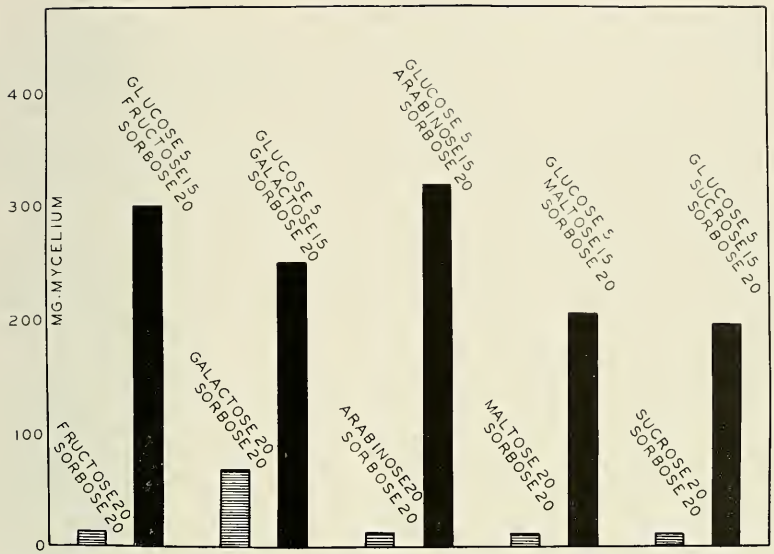

FIGURE 7. The effect of glucose in overcoming the inhibition of growth of Chaetomium globosum caused by sorbose. Time of incubation was 12 days, except for media containing L-arabinose where the time of incubation was 24 days.

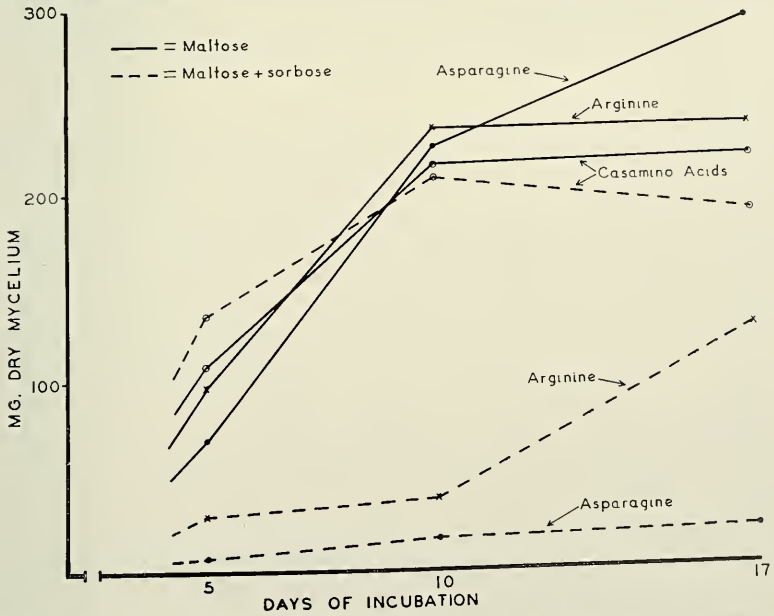

FIGURE 8. Growth of Chaetomium globosum in maltose and maltose-sorbose media containing different sources of nitrogen. Note that the inhibition due to sorbose is slight in Casamino Acids medium, intermediate in arginine and greatest when asparagine is used.

Some of our recent work with *Monascus purpureus* indicates that the nitrogen source is an important variable in studying the utilization of a single sugar. *Monascus purpureus* was cultured on sucrose media containing L-asparagine, L-arginine, and Casamino Acids as the source of nitrogen. The data are presented in Figure 9.

Another example of the effects of the nitrogen source on sugar utilization will be presented. *Monascus purpureus* was cultured in casein hydrolysate or yeast extract media containing glucose or maltose (Figure 10). The results obtained with the maltose-yeast extract medium should be noted since this substance is widely used in making media.

Concentration effects. The concentration of each and every constituent in a medium should be known if one hopes to duplicate results from experiment to experiment. In natural media, and host plants, neither the constituents nor their concentrations are known with any exactness. The effects of concentration in some laboratory experiments will be considered next.

It may be pointed out that the concentration of a vitamin needed by vitamin-dependent fungi depends on the concentration of the other constituents in the medium. *Ceratocystis fimbriata* requires an exogenous source of thiamine for growth and sporulation. For a given con-

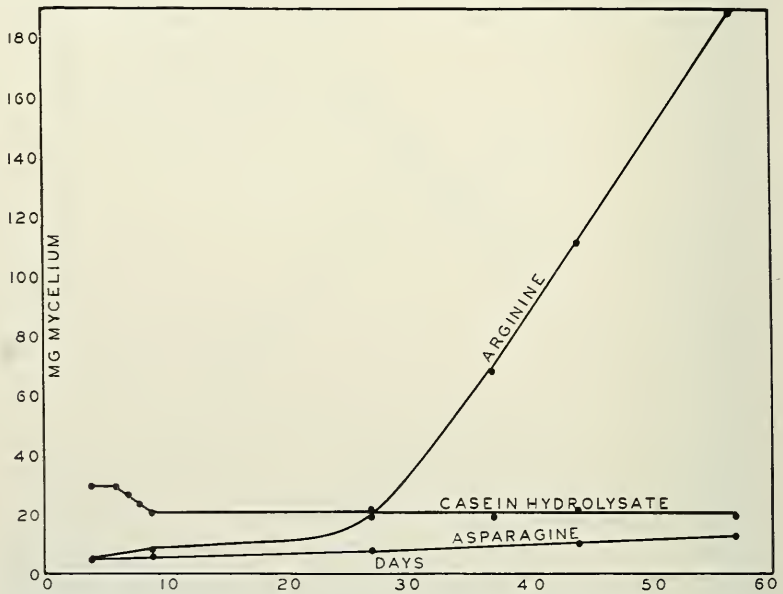

FIGURE 9. Growth of Monascus purpureus on sucrose media containing asparagine, arginine, and Casamino Acids as nitrogen sources.

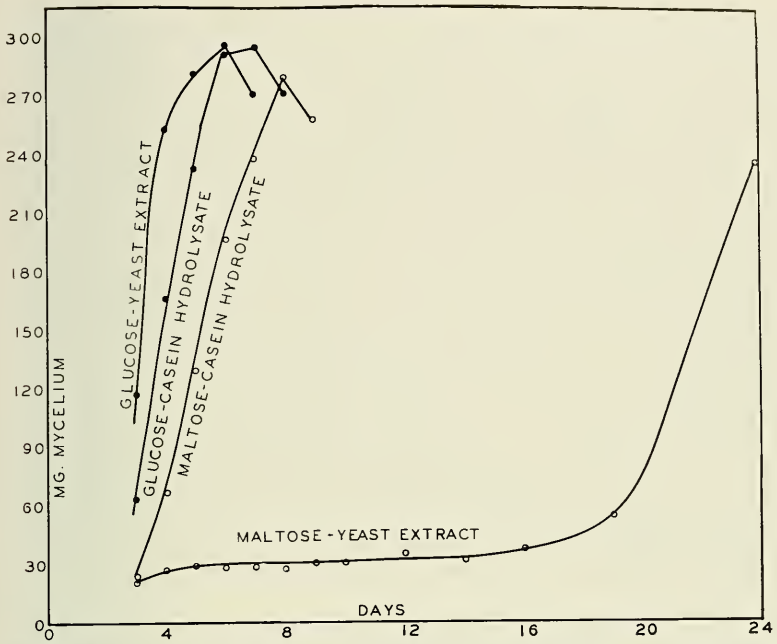

FIGURE 10. Growth of Monascus purpureus on glucose media containing yeast extract and casein hydrolysate as nitrogen sources, and on the same media when glucose was replaced by maltose.

centration of thiamine, the amount of growth and the formation of perithecia are related to the concentration of nutrients in the medium (Barnett and Lilly, 1947) (Figure 11).

Sordaria fimicola requires an exogenous supply of biotin for growth and sporulation (Lilly and Barnett, 1947; Barnett and Lilly, 1947a). When this fungus was cultured on media containing suboptimal concentrations of biotin it was found that both growth and the formation of ascospores, asci, and perithecia, were affected. Some of these effects are shown in Figures 12 to 14.

Effects of pH. All aqueous solutions have a pH value. Like temperature, pH effects are always present. In most laboratory experiments, no attempt is made to control the pH value of the medium during growth. Two techniques may be used to control the pH of a medium during incubation: (1.) By the use of a suitable internal indicator and the periodic addition of acid or base the pH of the medium may be kept relatively constant throughout the experiment. (2.) The flowing medium technique may be used. This method has the advantage that both the composition of the medium and the pH value may be maintained at desired levels. However, the method is tedious and exacting.

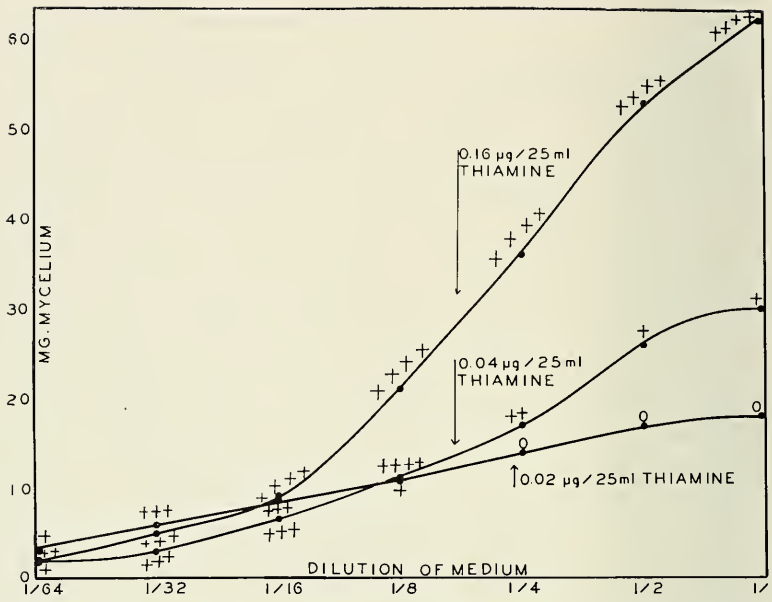

FIGURE 11. Growth and sporulation of Ceratocystis fimbriata as functions of the concentrations of nutrients and different concentrations of thiamine. Note that less thiamine is needed to support vegetative growth than the formation of perithecia and ascospores. (0, no perithecia; +, less than 20 perithecia/flask; ++, 20-200 perithecia/flask; +++, 200-1,000 perithecia/flask; ++++, more than 1,000 perithecia/flask. N.B. All perithecia contained mature ascospores.

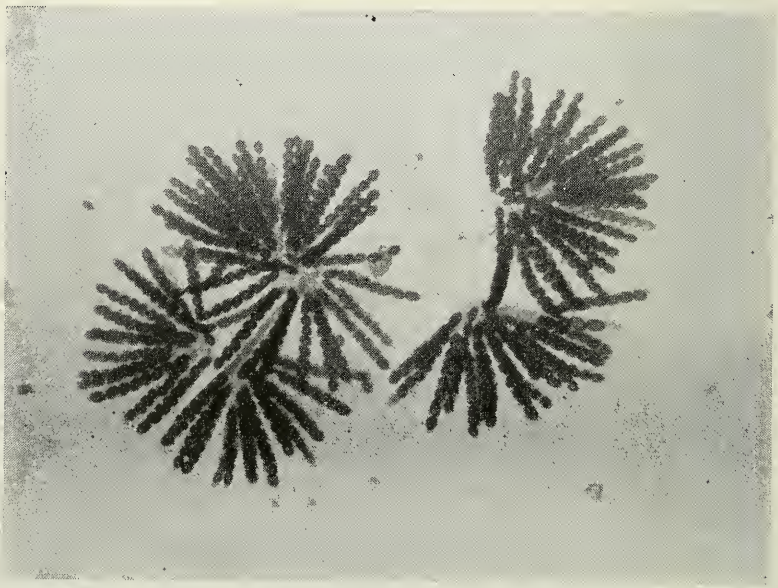

FIGURE 12. Group of normal asci from one perithecium developed on agar medium containing 6.4 µg/l of biotin.

FIGURE 13. Group of asci from a perithecium developed on agar medium containing 0.8 μg/l of biotin showing intermediate effects of biotin deficiency upon ascus and ascospore development.

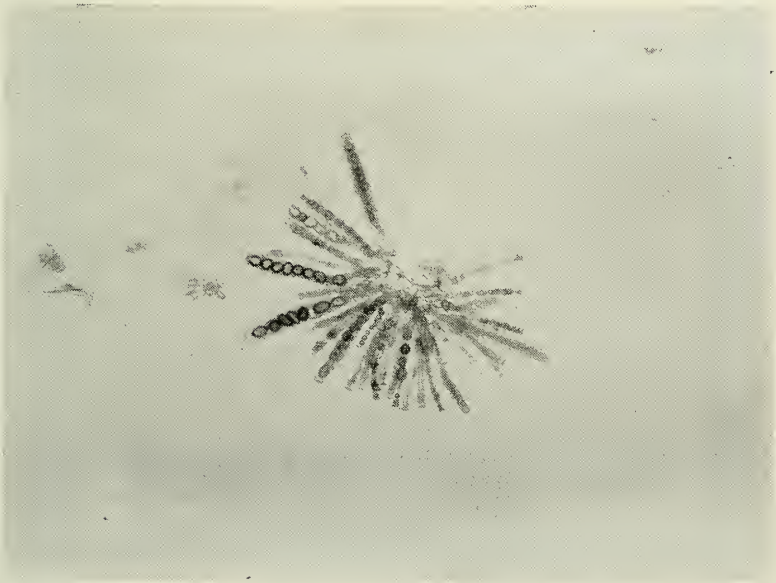

FIGURE 14. Group of asci from the same culture as Figure 12 showing severe effects of biotin deficiency. Note the high percentage of aborted asci and the few mature ascospores.

Barnett and Lilly (1956) devised the apparatus shown in Figure 15 to study the effect of pH and concentration of nutrients on the production of zygospores by *Choanephora cucurbitarum*. It had been found that no zygospores formed when ammonium sulfate or urea was used as nitrogen sources. In flask cultures, the pH of the culture filtrate fell to 3.1 with ammonium sulfate, and rose to 8.3 with urea. No zygospores formed with either medium. Was the failure of *C. cucurbitarum* to form zygospores in these two media due to the nature of the nitrogen sources or to the resulting pH values? By using the apparatus depicted above it was shown that the pH values were the responsible factors.

Media containing inorganic ammonium salts as nitrogen sources are commonly held in low esteem because many fungi grow poorly on them. Most fungi utilize ammonium-nitrogen, while about half of the species tested utilize nitrate-nitrogen. Leonian and Lilly (1940) found that the suitability of media containing ammonium nitrogen was greatly improved by adding certain organic acids.

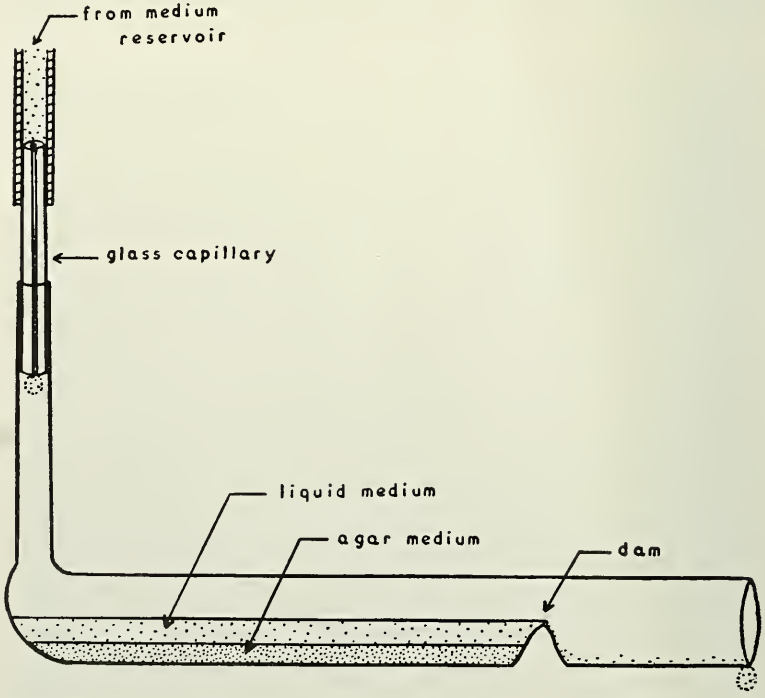

FIGURE 15. An apparatus for culturing fungi at constant pH.

We customarily use fumaric acid (2 g/l) when using inorganic ammonium salts as nitrogen sources. Lilly, Barnett, and Krause (1960) investigated the effects of adding varying amounts of potassium acetate to a basal glucose-ammonium sulfate medium on the growth, pH values of the filtrates, glucose utilization and carotene production by *Phycomyces blakesleeanus* (Figures 16 and 17).

Why should low pH values of the medium kill or inhibit the growth of fungi? It is natural to think that hydrogen ions are the toxic species. Indeed, this may be true in certain instances, but it will be shown that other factors may be equally important. It will be convenient to consider acetic acid which may be a metabolite or a toxic compound depending on the concentration (Lilly and Barnett, 1960). When acetate is added to a medium it gives rise to three species: undissociated acetic acid, acetate ion, and a cation. The concentration of undissociated acetic acid as a function of the acetate concentration and the pH of the medium is shown in Figure 18. The interrelated effects of pH and concentration of undissociated acetic acid on the growth of *Chaetomium globosum* are shown in Figure 19. *Fusarium lycopersici* behaved in the same way, except that it tolerated about twice the concentration of undissociated acetic acid as did *C. globosum* (Lilly and Barnett, 1961).

Wyss, Lilly, and Leonian (1944) found that the amount of p-aminobenzoic acid required to support the maximum growth of a mutant of *Neurospora crassa* increased as the pH of the medium increased (Figure 20). P-aminobenzoic acid is a weak organic acid like acetic acid. It would appear that only the undissociated acids enter the cells of fungi with any facility.

Pathogenic fungi produce a variety of toxins which are weak organic acids including gibberellic acid (*Fusarium moniliforme*), fusaric acid (*Fusarium* spp.), and alternaric acid (*Alternaria solani*). It would be expected that the toxicity of these and similar compounds would depend on the concentration of the undissociated acids, which in turn is determined by the concentration of the toxin and the pH of the substrate.

Methods of sterilizing media. The autoclave is efficient and quick, but the high temperature frequently results in the partial breakdown of reducing sugars such as glucose and fructose. This breakdown is particularly severe in the presence of phosphates. Some fungi make more growth when the sugar is autoclaved with the medium, others are inhibited by autoclaved medium (Margolin, 1942).

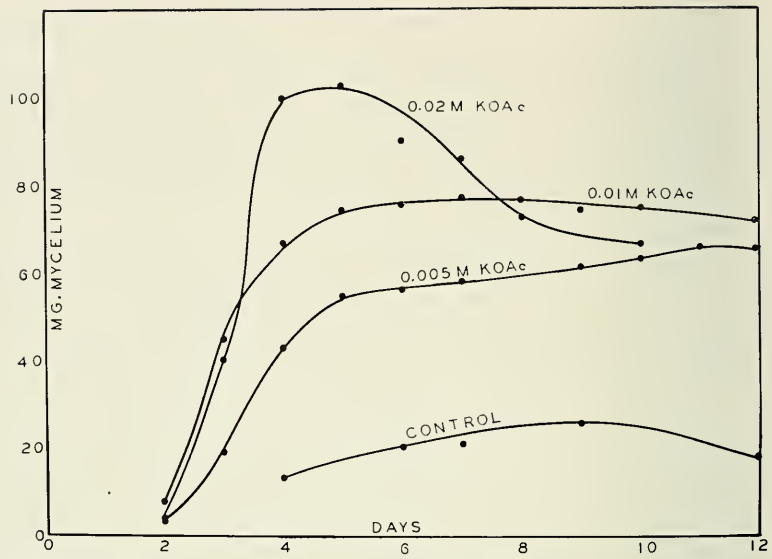

FIGURE 16. The effect of adding 0, 0.005, 0.01, and 0.02 M potassium acetate to a glucose-amonium sulfate medium on the growth of Phycomyces blakesleeanus.

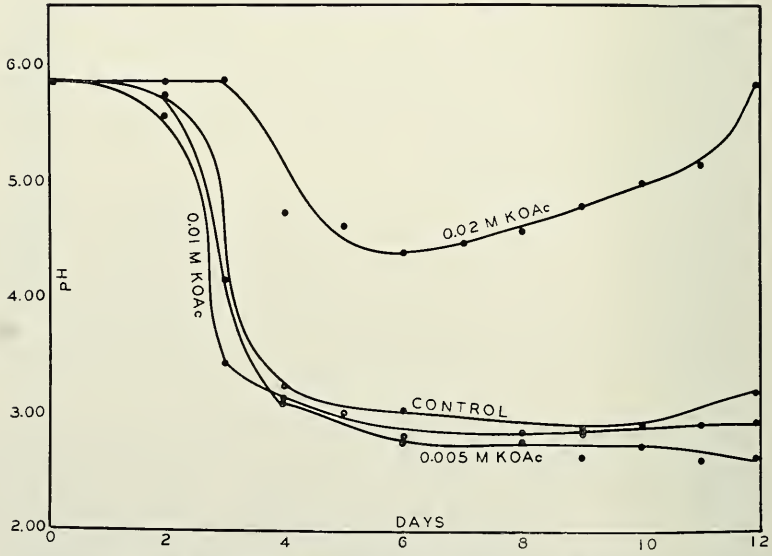

FIGURE 17. The effect of adding 0, 0.005, 0.01, and 0.02 M potassium acetate to a glucose-ammonium sulfate medium on the changes in pH values due to the growth of Phycomyces blakesleeanus.

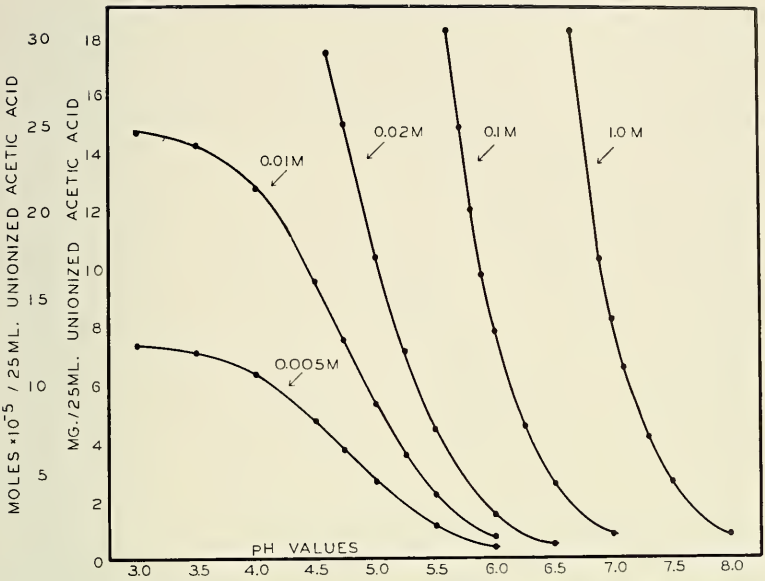

FIGURE 18. The concentration of undissociated acetic acid as a function of the pH value and the concentration of acetate.

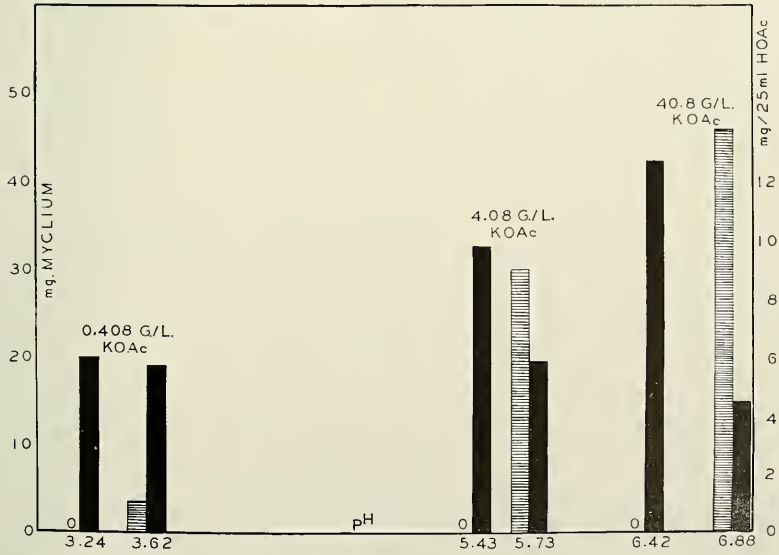

FIGURE 19. The effect of pH and the concentration of undissociated acetic acid on the growth of Chaetomium globosum.

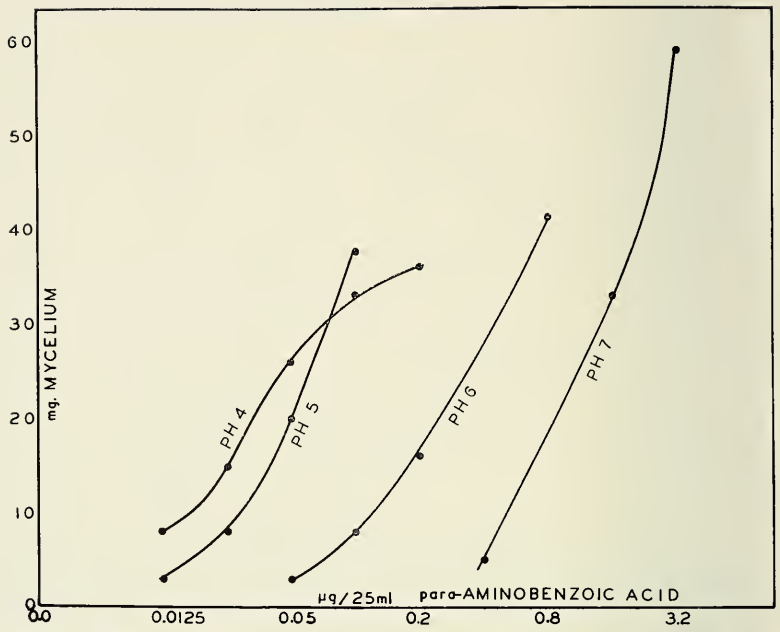

FIGURE 20. Growth of a para-aminobenzoic acid-deficient mutant of Neurospora crassa **as a function of the pH value of the medium and the concentration of para-aminobenzoic acid.**

Barnett, Lilly, and Morris (1953) compared the growth of *Thielaviopsis basicola* on media containing glucose and fructose. Half of each medium containing the sugars was autoclaved, while the sterile filtered sugars were added to the autoclaved basal medium. The cultures were incubated at 20, 25, 30, and 31.5° C (Figures 21 and 22). Wilson (1954) grew *T. basicola* on autoclaved and filtered sucrose (Figure 23).

It may be noted that temperature plays a more important role in the toxicity of autoclaved fructose than in autoclaved glucose. Characteristically, fructose media darkens more on autoclaving than glucose media. Note also that fructose supported more growth than glucose, particularly at 30 and 31.5° C. This incomplete set of data suggests that the upper temperature limit for *Thielaviopsis basicola* depends on the sugar used in the medium and the method of sterilization.

Methods of inoculation. Agar media are frequently used to study sporulation. For physiological and epidemiological studies it is desirable to produce quickly large numbers of spores which are of uniform age and properties. For some species, a suitable procedure consists of flooding the agar surface with a dense suspension of spores or of blended mycelium.

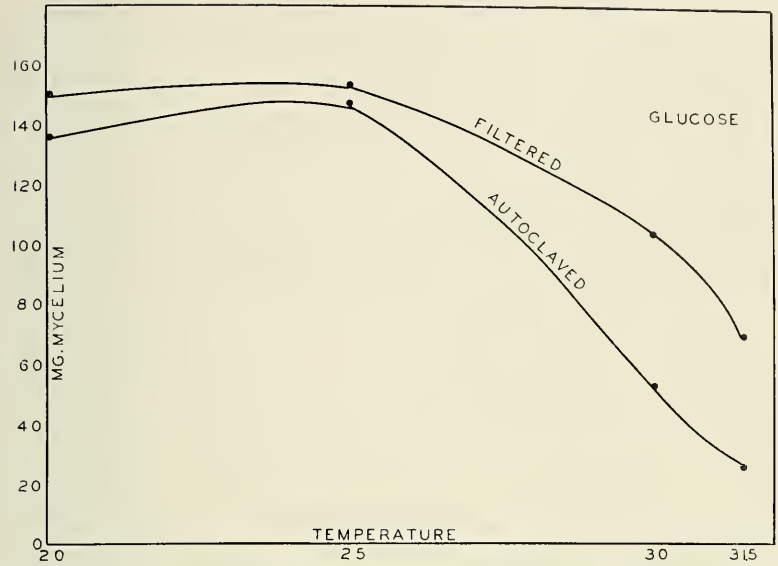

FIGURE 21. Growth of Thielaviopsis basicola on media containing autoclaved and filtered glucose, eight days.

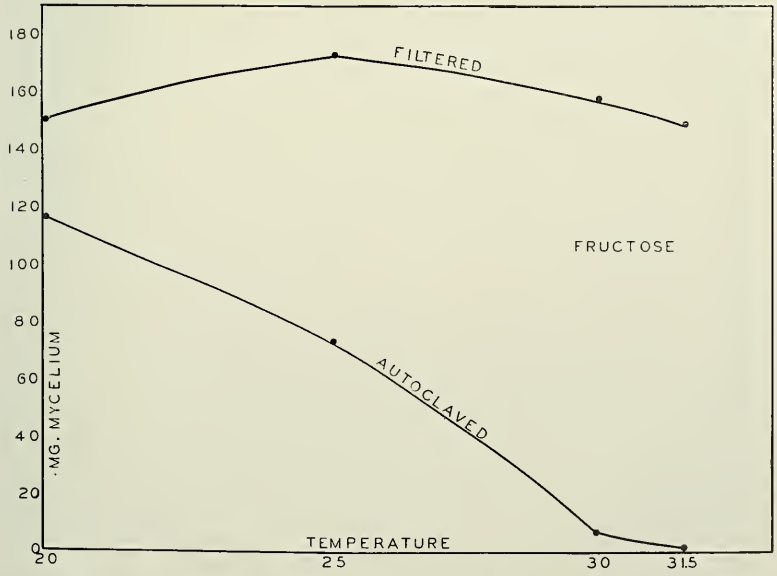

FIGURE 22. Growth of Thielaviopsis basicola on media containing autoclaved and filtered fructose, eight days.

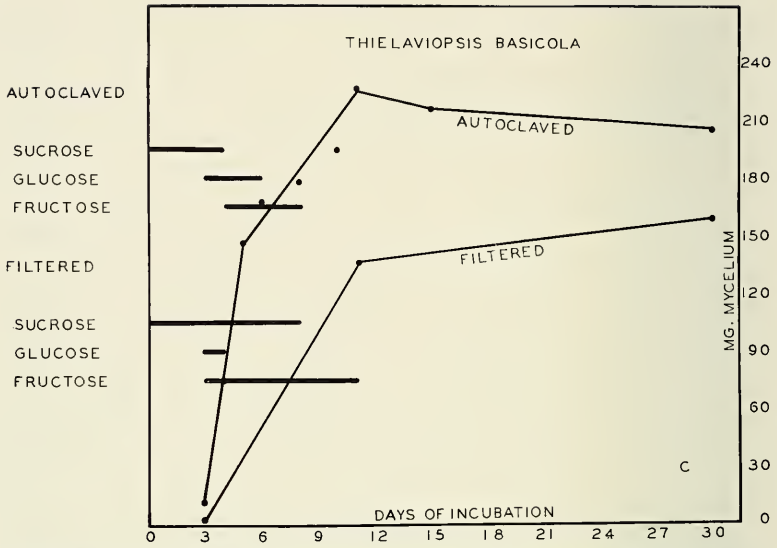

FIGURE 23. Growth of Thielaviopsis basicola on media containing autoclaved and filtered sucrose. The bars designate the days during which sucrose and its hydrolytic products were detected in the culture filtrates by paper chromatography.

Timnick, Lilly, and Barnett (1951) and Timnick, Barnett, and Lilly (1952) compared the rate and amount of sporulation of *Melanconium fuligeneum* on agar medium in which the plates were inoculated in the center with a bit of mycelium or a drop of spore suspension and by flooding the agar surface with spores or cut mycelium. In plates inoculated by the point method, the mycelium reached the edge of the dish within 5 to 6 days, sporulation began between the 11th and 14th day; sporulation reached a maximum (1.2×10^9 spores/plate) after 20 days of incubation. Under these conditions, the spores were produced in large acervulus-like hyphal masses (Figure 24). This is the normal sequence of events for many fungi grown from a point inoculum. When the plates were flood-inoculated, the colony covered the agar surface from the time the cut mycelium began to grow, or the spores to germinate. The surface was covered with mycelium of uniform age. These cultures began to produce spores within 24 hours; the maximum number of spores (2.5×10^9/plate) was produced within four days. No acervulus-like structures were produced (Figure 25). Instead, spores were formed abundantly on germ tubes distributed along the hyphae (Figure 26). Widely separated spores on germination produced mycelium as shown in Figure 27. It would appear that rapid exhaustion of nutrients was the probable cause of rapid sporulation, a factor known to favor, or be required for the sporulation of many

species. This hypothesis was supported by the results obtained from experiments in which the plates were flooded with tenfold serial dilutions of spores. The higher the dilution, the longer it took for sporulation.

The flooded-plate technique works well with many but not all species. As a further example, Figure 28 shows the appearance of point inoculated *Glomerella cingulata* culture and Figure 29 the appearance of a culture inoculated by flooding with spores.

Effects of environment. Examples of the effects of temperature and light will be presented.

Temperature. Laboratory cultures are usually grown at fixed temperatures; in the field fluxuating temperatures are the rule. More laboratory studies are needed in which fungi are cultured under a programmed temperature cycle. The upper temperature limit has been reported in some instances to be affected by the medium. Barnett and Lilly (1948) studied the vitamin requirements of an isolate of *Sclero-*

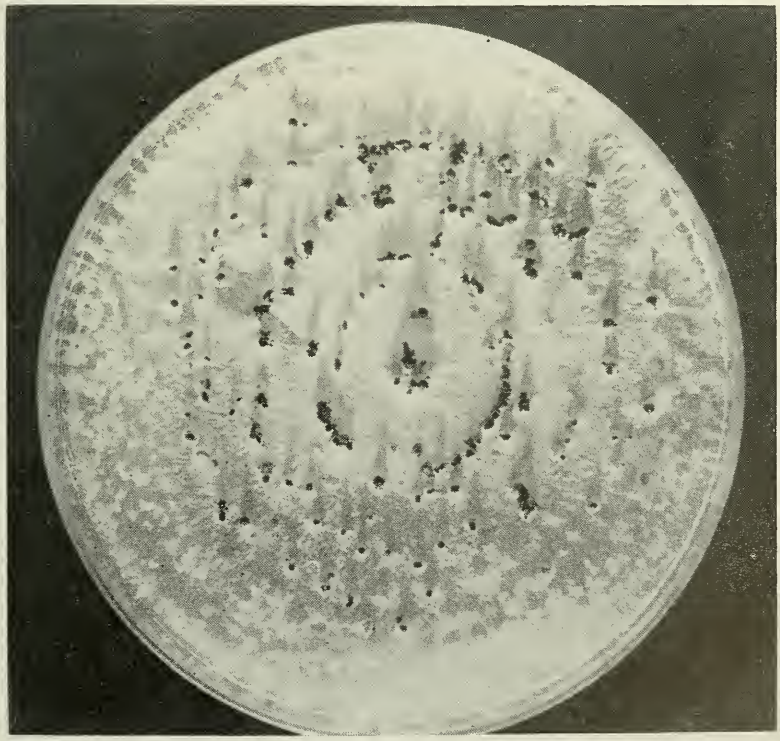

FIGURE 24. Melanconium fuligeneum, point inoculated, 20 days.

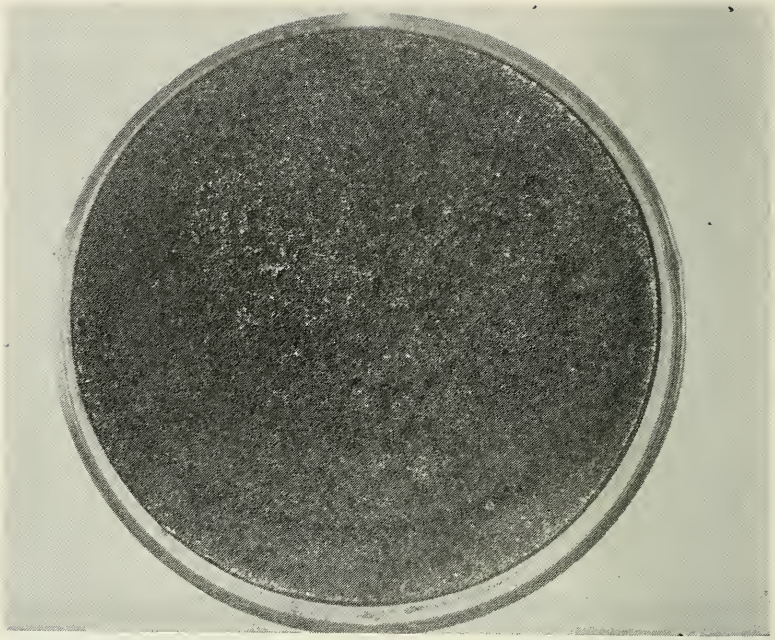

FIGURE 25. Melanconium fuligeneum. Flood inoculated, four days.

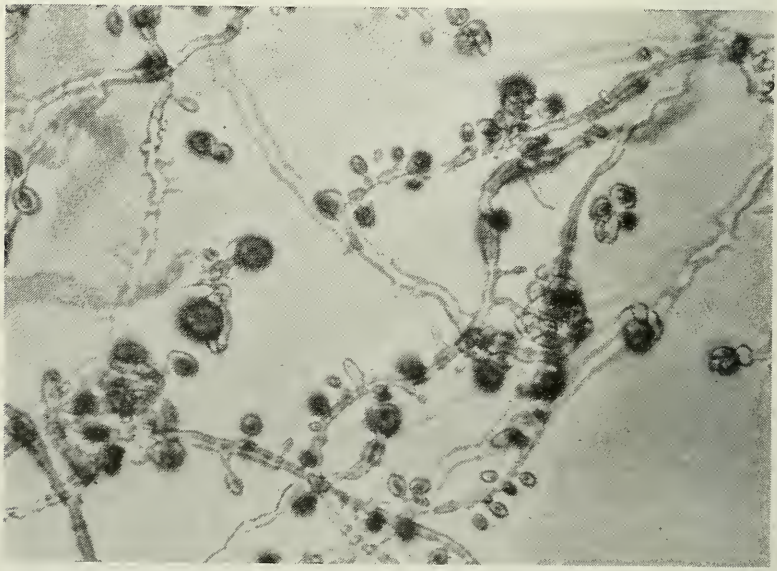

FIGURE 26. Melanconium fuligeneum. Method of spore production with flooded-plate technique, four days.

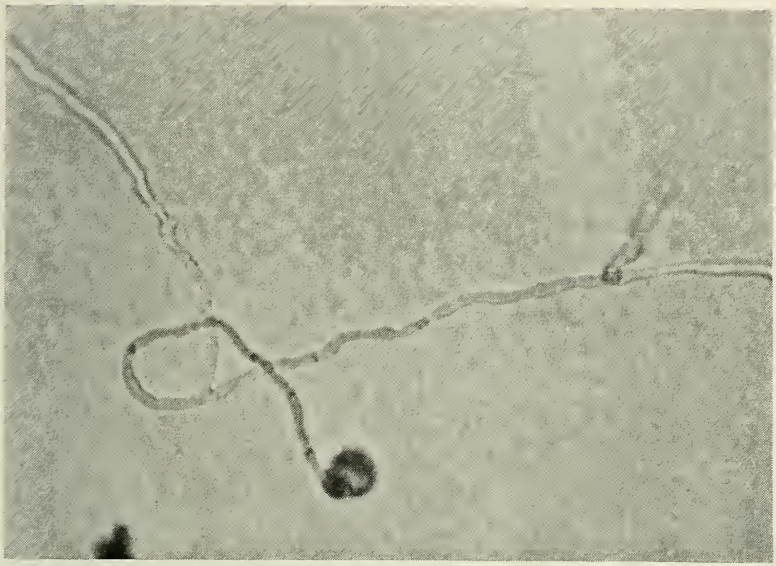

FIGURE 27. Germinating spore of Melanconium fuligeneum. Note that mycelium, but no spores are formed when the germinating spores are far apart.

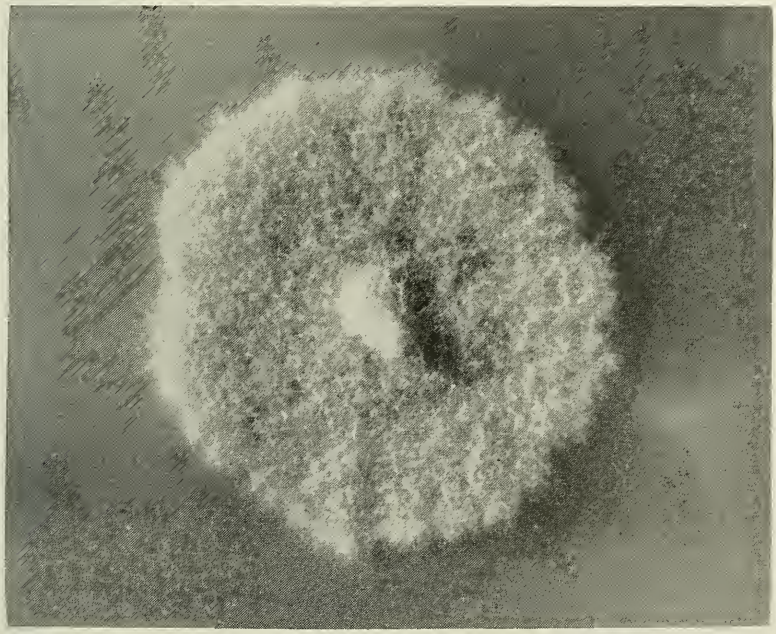

FIGURE 28. A point inoculated culture of Glomerella cingulata.

FIGURE 29. A portion of a plate flood inoculated with Glomerella cingulata.

tinia camelliae and found that thiamine and biotin were required at all temperatures tested. The addition of inositol increased the amount of growth at the lower temperatures so it was thought that this isolate synthesized insufficient inositol to allow the maximum rate of growth. The effects of temperature and inositol concentration were then studied in more detail (Figure 30).

The effect of the medium on the rate of growth at different temperatures is seen when *Monascus purpureus* is grown in glucose-asparagine and maltose-asparagine media at 20, 25, and 30° C (Figures 31 and 32).

The above data illustrate the supreme importance of another variable, time. This utilization of maltose at 20° C would probably have been overlooked had the experiment been terminated when the maximum dry weight had been produced on glucose.

Light. It is recognized that many fungi require light for sporulation. The compounds that act as light acceptors and the mechanism of action are unknown. *Choanephora cucurbitarum* not only requires light for asexual sporulation, but also darkness (Barnett and Lilly, 1950). Indeed, the mycelium must be exposed to light and then darkness before conidia form (Figure 33). Light has no apparent effect on zygospore formation by this species.

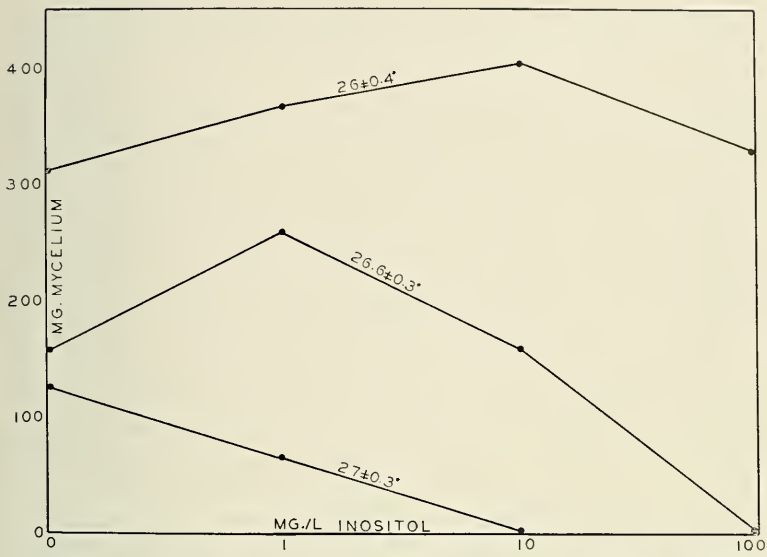

FIGURE 30. The effect of temperature and concentration of inositol on the growth of Sclerotinia camelliae.

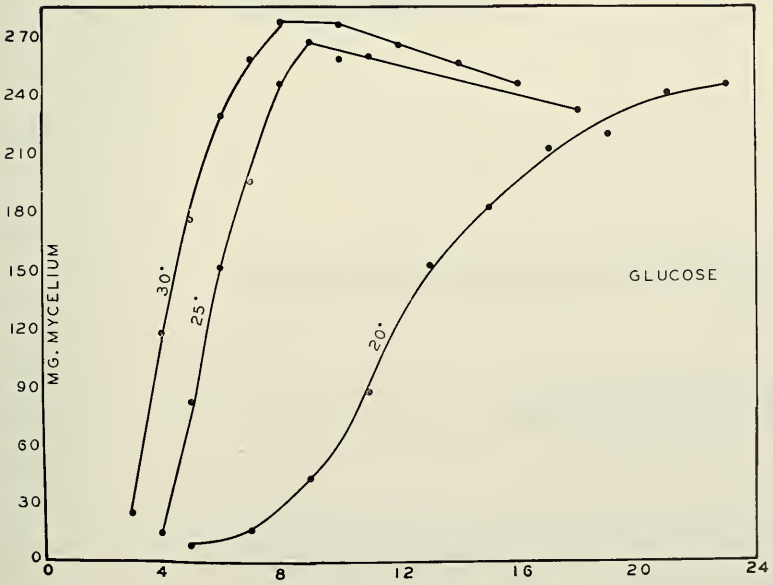

FIGURE 31. The effect of temperature on the growth of Monascus purpureus in glucose-asparagine medium.

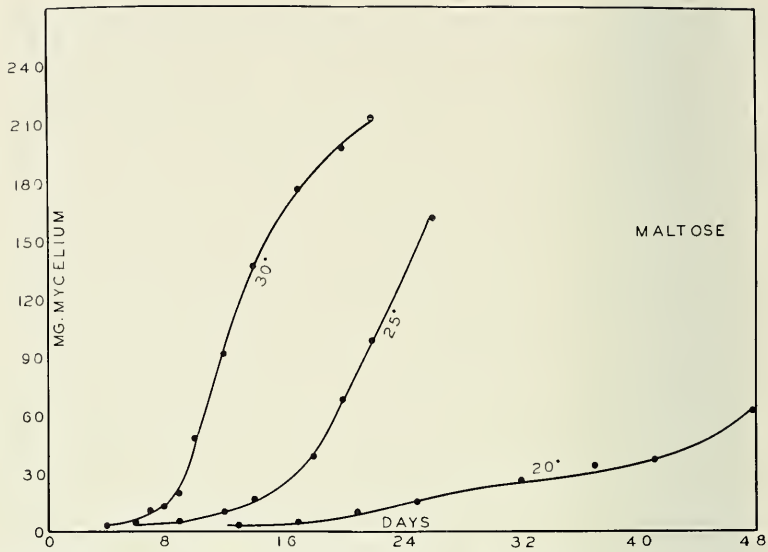

FIGURE 32. The effect of temperature on the growth of Monascus purpureus in maltose-asparagine medium.

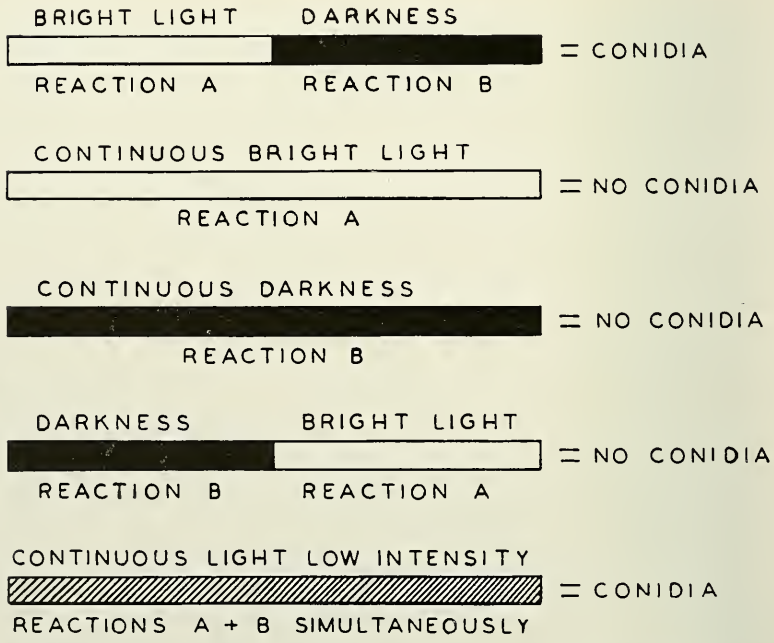

FIGURE 33. Conidium formation by Choanephora cucurbitarum under different light conditions.

Recently, we have observed that continuous light (ca. 60 ft-c) depresses the rate and amount of growth of *Choanephora cucurbitarum*. Light also depresses the rate of glucose utilization (Figure 34).

Measuring growth. Two methods of measuring growth in the laboratory are in common use. (1) The fungi are cultured on agar medium and the diameter of the colony is measured. The simplicity of this method recommends itself to many. It has the advantage that the same colonies may be observed repeatedly. (2) The dry weight of the mycelium produced may be determined. Either liquid or agar media may be used. Both methods of measuring growth are objective.

Are the results obtained by using these two methods of measuring growth comparable and will the same conclusion be reached irrespective of the method used to measure growth? At various times we have measured growth of the same fungi using these two methods. *Glomerella cingulata* was grown on four media at the same temperature in petri dishes and 250-ml Erlenmeyer flasks. Growth was measured after 3, 6, 9, and 12 days. The data for linear growth are given in Figure 35 and for dry weight of mycelium in Figure 36.

If the diameter of the colonies be used as the criterion of growth, it will be concluded that *Glomerella cingulata* makes essentially the same amount of growth on glucose and lactose, and strangely enough,

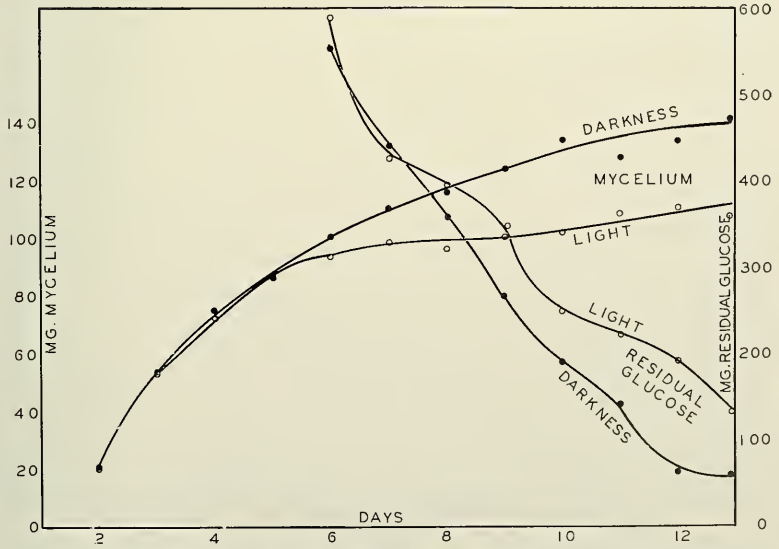

FIGURE 34. Growth and glucose utilization of Choanephora curcurbitarum **in light and in darkness.**

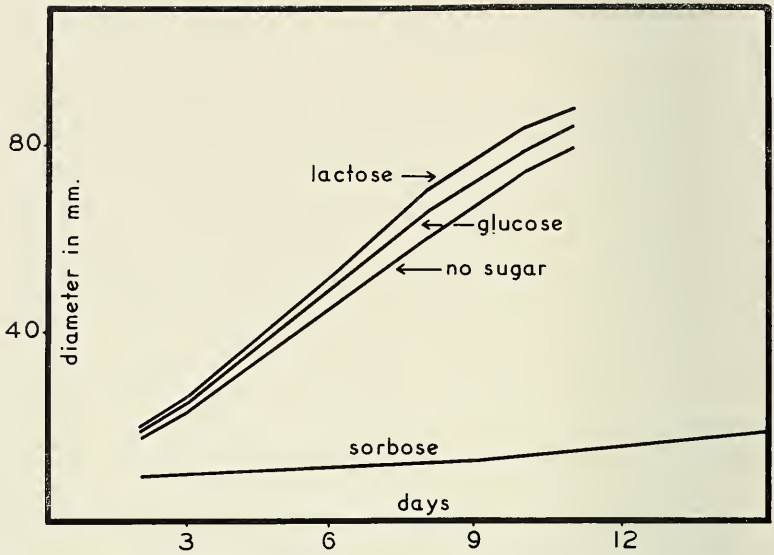

FIGURE 35. Growth of Glomerella cingulata measured as diameter of colonies.

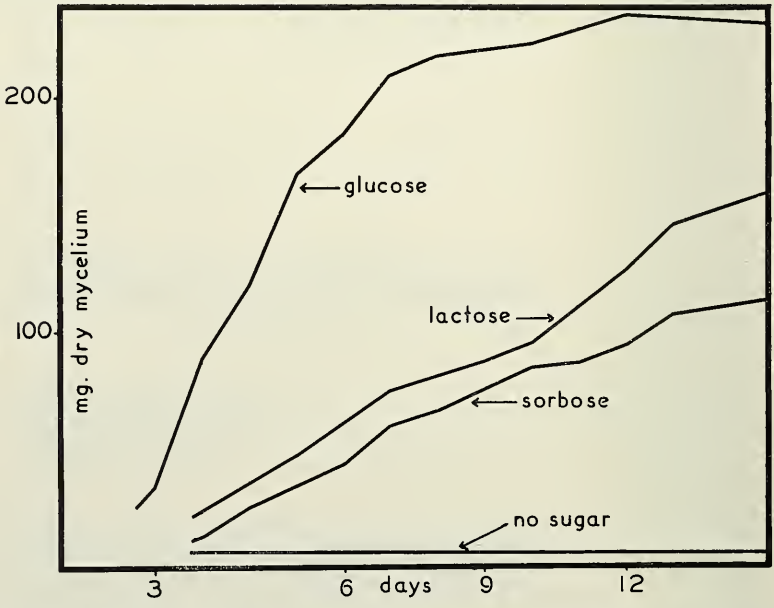

FIGURE 36. Growth of Glomerella cingulata measured as dry weight of mycelium.

neither of these sugars increase growth above that obtained in the basal medium without added sugar. L-sorbose, according to this criterion of growth, supports less growth than the control medium.

If the dry weight of mycelium produced be used as the criterion of growth it will be concluded that about twice as much growth occurred on glucose as occurred on lactose or sorbose. Only a trace of growth occurred on the sugar-free medium. Evidently, the method used to measure growth influences the results. Are these results typical, or do they apply to only *Glomerella cingulata*? On the basis of many unpublished results in which the concentration of a single sugar was varied, vitamin concentrations, and different sources of nitrogen were used, it was concluded that colony diameters are a highly unsatisfactory method of measuring growth.

It will be noted that the linear method of measuring growth was criticized, not the use of agar media for certain purposes. Agar media are in constant use in our laboratories, particularly for studying sporulation. I wish to point out that it is perfectly feasible to determine the weights of mycelium produced on agar medium. The technique (Timnick, Lilly, and Barnett, 1951) consists of adding a little water to the petri dish cultures, melting the agar at 100° C, and washing the mycelium with hot water. The mycelium is then dried and weighed in the usual manner. Some soluble material is removed by this treatment so the weights are not comparable to those from liquid medium.

Ceratocystis fimbriata and *Aspergillus rugulosus* were grown on a Casamino Acid basal medium, the glucose concentration was varied. The data for diameter and for dry weights of mycelium of the *same* cultures are given in Figures 37 and 38.

Variation. The fungi are variable; different isolates of a given species vary in physiology, and pathogenicity. The plant pathologists recognize variability when they speak of such fungi as *Puccinia graminis* var. *tritici* race 15, or *P. graminis* var. *avenae,* or *Fusarium solani* f. *pisi,* or *F. solani* f. *phaseoli,* or *Phytophthora infestans* potato race 0, or *P. infestans* potato race 1, 4, etc. These long designations are necessary to distinguish isolates, races, from species which differ in pathogenicity to specific hosts. While the "lumpers" in taxonomy reduce the number of binomial epithets, this loss may be compensated for by increasing the number of varieties, races, strains, and form species. Alexopoulos (1952), it seems to me, was correct in remarking that we work with individuals and their decendants. Should this view be correct, we would expect to find that, when the same taxonomic species is studied in different laboratories, or different isolates are studied in the

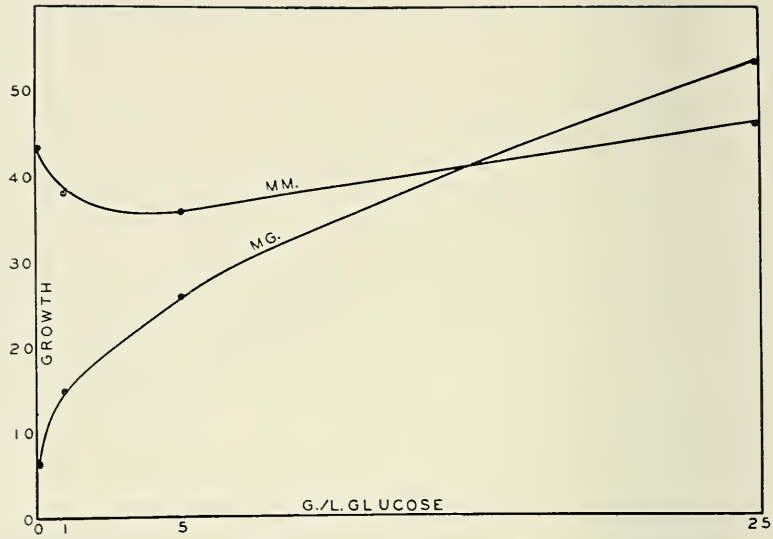

FIGURE 37. Growth of Ceratocystis fimbriata measured as diameter and as dry weight of mycelium, five days. The same cultures were used for both determinations.

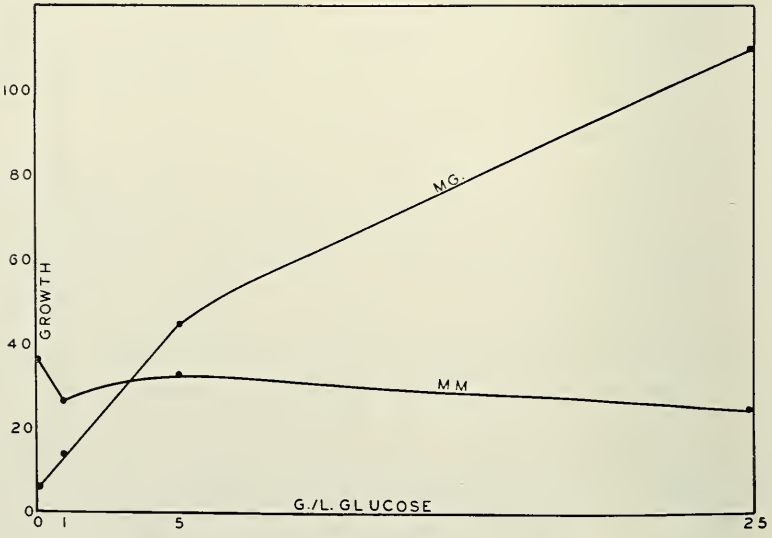

FIGURE 38. Growth of Aspergillus rugulosus measured as diameter and as dry weight of mycelium, 11 days. The same cultures were used for both determinations.

same laboratory under the same conditions, differences in physiology will be observed.

This laboratory has been studying the pathogen of oak wilt since 1952. Numerous morphological and physiological variations have been observed among isolates obtained from infected oak trees. Albino and dark isolates, unisexual males and other variants have been found. The response of ten isolates of *Ceratocystis fagacearum* to added thiamine and biotin is shown in Figure 39 (Barnett, 1955). Note that no two of the ten isolates were identical.

Is the observed variation due to the fungus, or to minute changes in the medium or the environment? Fungi differ in variability. We have conducted extensive studies of two fungi, *Phycomyces blakesleeanus* and *Choanephora cucurbitarum* during the past few years. The weights of duplicate cultures of *P. blakesleeanus* seldom differ by more than 2 mg, while duplicate cultures of *C. cucurbitarum* frequently differ by as much as 10 mg.

However, before attributing all of the observed variation to the fungus it should be pointed out that seemingly minute changes in the procedure may affect a fungus in unexpected ways. For many years we have been studying the production of carotene by *Phycomyces blakesleeanus*, using this fungus to produce *beta*-Carotene-U-C^{14} (Lilly, Barnett, Krause, and Lotspeich, 1958; Lilly, Barnett, and Krause, 1960). Some runs deviated widely from the average, particularly during the

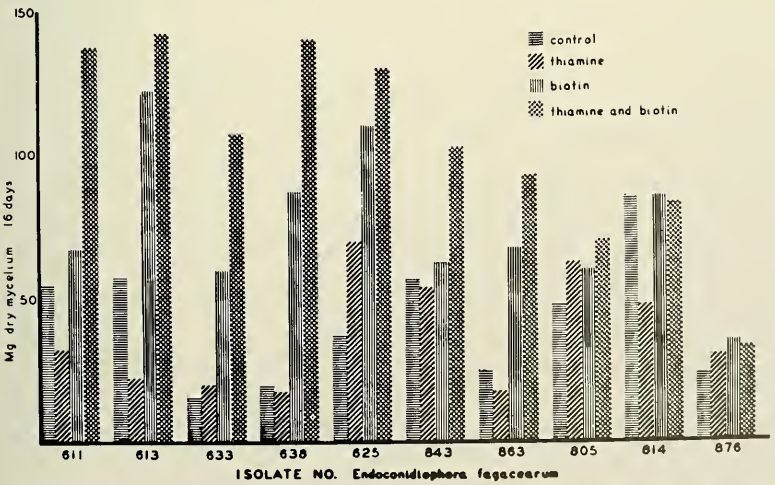

FIGURE 39. Growth of ten isolates of Ceratocystis fagacearum **under identical conditions. Note that no two of the ten isolates were identical.**

first few days of incubation. Two factors were shown to be involved. The first concerned the method of adjusting the pH of the medium before autoclaving. Sodium hydroxide and hydrochloric acid were used; we had not considered that the sodium chloride formed would affect *P. blakesleeanus*. Sodium chloride did inhibit germination of freshly harvested sporangiospores (Figure 40). This was not the entire story for it was found that the sensitivity of the spores to sodium chloride depended on their age after harvesting and the temperature at which they were produced (Lilly, Barnett, and Krause, 1960; Lilly, Barnett, and Krause, 1962).

DISCUSSION

To sum up, fungi are extremely sensitive to changes in nutrition and environment. If it is desired to reproduce the same results in repeated experiments great care must be exercised in maintaining the same nutritional and environmental conditions. Minor changes in nutrition and environment may result in drastic changes in the activities of a fungus. No change in composition of medium, the environment, or procedure, can safely be assumed to be without effect.

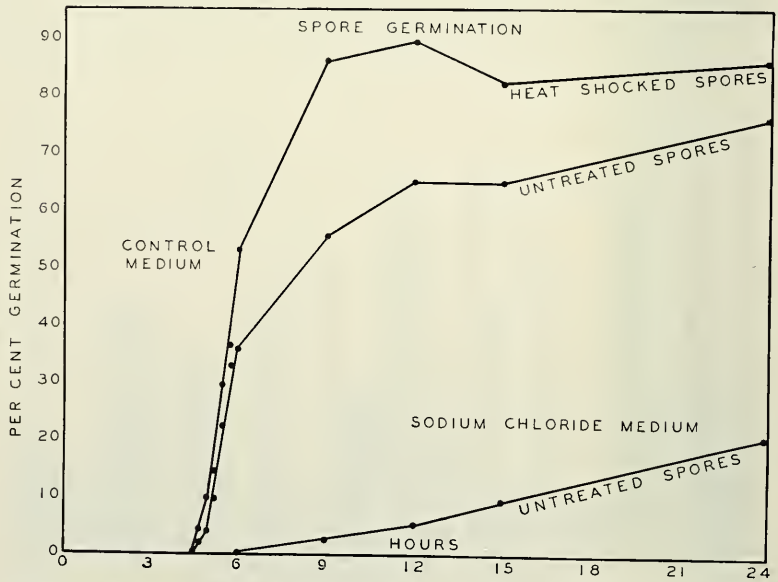

FIGURE 40. The effect of adding 0.5 g/l of sodium chloride to a glucose-ammonium sulfate medium in the germination of sporangiospores of Phycomyces blakesleeanus.

There is no reason to assume that the same or similar effects do not occur in the field. Indeed, it would be expected that a larger number of variables are encountered when a fungus pathogen is living in or on its host in the field than in the laboratory. The lesson to be learned from fungus physiology is not so much answers, but the questions to be asked.

May I close with two quotations? The first is from the Priestly Medalist's lecture by Hildebrand (1962). "The prime requirement for dealing with an opponent is intelligence. I use that term both in its primary meaning and also the one I learned in the Army." The second is an aphorism of the Father of Medicine and a profound student of disease physiology: "Life is short and art is long, the occasion fleeting, experience fallacious and judgement difficult" (Haggard 1929).

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The Physiology of Mycoparasitism

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PARASITISM is a mode of life in which many heterotrophic organisms, under appropriate conditions and in close association with other living organisms of a different species, are able to absorb all or part of their essential nutrients from their associates, the hosts. The best known parasites are those of economic plants and animals, whereas relatively little is known about the fungi that parasitize other fungi. The term *mycoparasitism* was coined by Butler (1957) to designate the parasitic relationship between two fungi. The purpose of this review paper is to bring together the information on the physiological relations of mycoparasitism, with emphasis on the recent studies made in our laboratory at West Virginia University.

Mycologists and plant pathologists have not utilized to full advantage the mycoparasites as tools in the study of basic principles of parasitism. These organisms offer certain advantages in savings of time and space over the parasites of higher economic plants. The interested investigator has a wide choice of fungi for study.

The greatest number of known species of mycoparasites belong to the Chytridiales, Mucorales, and the Imperfects. Only a few are members of the Saprolegniales or Peronosporales. Intrageneric parasitism is known to occur only in the Chytridiales (Karling, 1960), but it would not be surprising to find it elsewhere. With only three known exceptions, species of mycoparasitic Mucorales also have other Mucorales as their hosts. According to Tubaki (1955), more than 220 species of Hyphomycetes alone, in over 69 genera, have been reported to be fungicolous, but it is not known how many of these are truly parasitic.

Based on spatial relationship, the mycoparasites may be primarily external or internal. Some Chytridiales are entirely internal, whereas in others only the rhizoids are internal. True haustoria are produced by some Mucorales. Many mycoparasites produce unspecialized hyphae which penetrate the host hyphae and grow internally. Others are completely external, making close contact with their hosts by means of special branches or entwining hyphae. Intermediate and combinations of types may also be found.

For convenience, the mycoparasites may be classed in two general groups: (1) *The destructive mycoparasites*, which kill and destroy all or part of the host by means of enzymes or toxic substances; and (2) *The balanced mycoparasites*, that cause little damage to the host, which in turn continues to live and furnish the parasite with required nutrients. The term "obligate parasite" does not seem appropriate here, because with our present knowledge several species formerly believed to be obligate mycoparasites can now be cultured axenically under special nutritional conditions. Among the first studies contributing to this knowledge were those of Ayers (1933, 1935), who found that *Dispira cornuta* could be grown in the absence of the host on natural media rich in nitrogen.

The study of mycoparasitism in the Department of Plant Pathology, Bacteriology, and Entomology was initiated about 1954, following the discovery of a species of *Piptocephalis* and one of *Calcarisporium*, both of which are balanced mycoparasites. Since 1955 the work has become intensified and expanded to include mycoparasites of other types and species. It has been supported, in part, by grants from the National Science Foundation, in conjunction with broader physiological studies on physiology of fungi in the West Virginia University Agricultural Experiment Station. The investigations have been carried on mainly by graduate research assistants. Publications reporting the results of this work are listed in the literature citations.

DESTRUCTIVE MYCOPARASITES

Parasites destructive to Rhizoctonia solani: One of the first fungi to be incriminated as a parasite (and pathogen) of other fungi was *Trichoderma lignorum*. Weindling (1932) reported that the hyphae of *T. lignorum* attacked hyphae of *Rhizoctonia solani* and other soil fungi and caused rapid destruction of the host cells. An additional antibiotic action was discovered when it was shown that an extract from a culture of *T. lignorum* checked the growth of other fungi.

Warren (1948) added to our knowledge of this mode of parasitism when he discovered a new species of *Papulospora* parasitizing and rapidly destroying the hyphae of *Rhizoctonia solani*. Death of host cells occurred only after close contact by coils or penetration by parasite hyphae.

A few years later, Boosalis (1956) reported that *Penicillium vermiculatum* penetrated the walls of *Rhizoctonia solani* and made sparse growth within the cells, causing deterioration of the protoplasm. Extracts of the mycelium of this parasite failed to reduce growth of the

host. Parasitism was greatest on an agar medium containing 20 g glucose per liter and was negligible when there were 10 g per liter.

In the studies cited above the investigators were interested in the possible use of parasitic organisms in the biological control of *Rhizoctonia solani* in the soil. It is not surprising, therefore, that little physiological work on the parasite-host relationship was included.

Rhizoctonia solani as a mycoparasite: A "new twist" was revealed when Butler (1957) discovered that *Rhizoctonia solani* can act in turn as a parasite of other soil fungi. Of the 20 isolates of *R. solani* obtained from various sources and tested for parasitism on *Phythium debaryanum* and *Rhizopus nigricans*, 6 were parasitic on both, 3 on the former host only, 5 on the latter only, and 6 were nonparasitic. Species of *Pythium* were parasitized by coiling type of mycelium, but species of the Mucorales and *Amblyosporium botrytis* were parasitized by both coiling and penetrating mycelium.

Examples of both mechanical and physiological resistance were observed by Butler. Infection hyphae were frequently sealed off by a protective sheath of wall-like material. In other cases, internal mycelium of the parasite was seen to break down almost immediately after entry into the host.

The fact that parasitism was severe on potato-glucose agar and weak or absent on water agar suggested that the composition of the medium was an important factor. Using a nitrate-salts basal medium, it was shown that the carbon source greatly influenced vegetative growth of both host and parasite and the degree of parasitism. Butler reached two general conclusions: (1) that parasitism depended upon a balance of nutritional and environmental factors; and (2) that good vegetative growth of both host and parasite was prerequisite to infection. It is also evident that other metabolic factors affect susceptibility, for poor parasitism occurred even though both host and parasite made good vegetative growth on xylose, galactose, cellobiose, and glycogen. Furthermore, susceptibility was higher when both hosts and parasites were grown in darkness than when grown in continuous light.

Gliocladium roseum as a parasite: Although *Gliocladium roseum* is frequently found in nature associated with other fungi, there was little evidence of its parasitic nature until Shigo (1958) reported this species overgrowing and destroying mats of the oak wilt fungus. Tubaki (1955) reported *G. roseum* as a fungicolous species but gave no evidence of parasitism.

The destructive parasitic activities of *G. roseum* on numerous fungi have since been observed in our laboratory (Barnett and Lilly,

1962). The mode of parasitism is similar to that of *Papulospora stoveri* and of *Rhizoctonia solani,* except that there is less twining of the parasite hyphae around the hosts. The destructive activities of *G. roseum* were frequently more intense on spores than on vegetative cells of the host.

On a suitable agar medium, such as glucose-yeast extract, *G. roseum* overgrew both young and established colonies of numerous species of host fungi in various taxonomic groups. One of the most susceptible species was *Ceratocystis fimbriata,* which was completely destroyed in culture within ten days. Parasite hyphae growing near spores of *C. fimbriata* sent out short branches which touched and often looped around the cell. Only the spores directly in contact with the parasite were killed immediately with rapid disintegration of the protoplasm (Figures 1-3). The killed spores of some hosts, such as *Trichothecium roseum,* were penetrated and later often showed disintegration of the walls (Figures 4-6). Hyphal cells and conidia of *Rhinotrichum macrosporum* were killed and sometimes penetrated (Figures 7-10). On the other hand, *Helminthosporium sativum* was highly resistant, only immature conidia and conidiophores being destroyed (Figures 11-13). Penetration of living host cells by *G. roseum* seldom occurred.

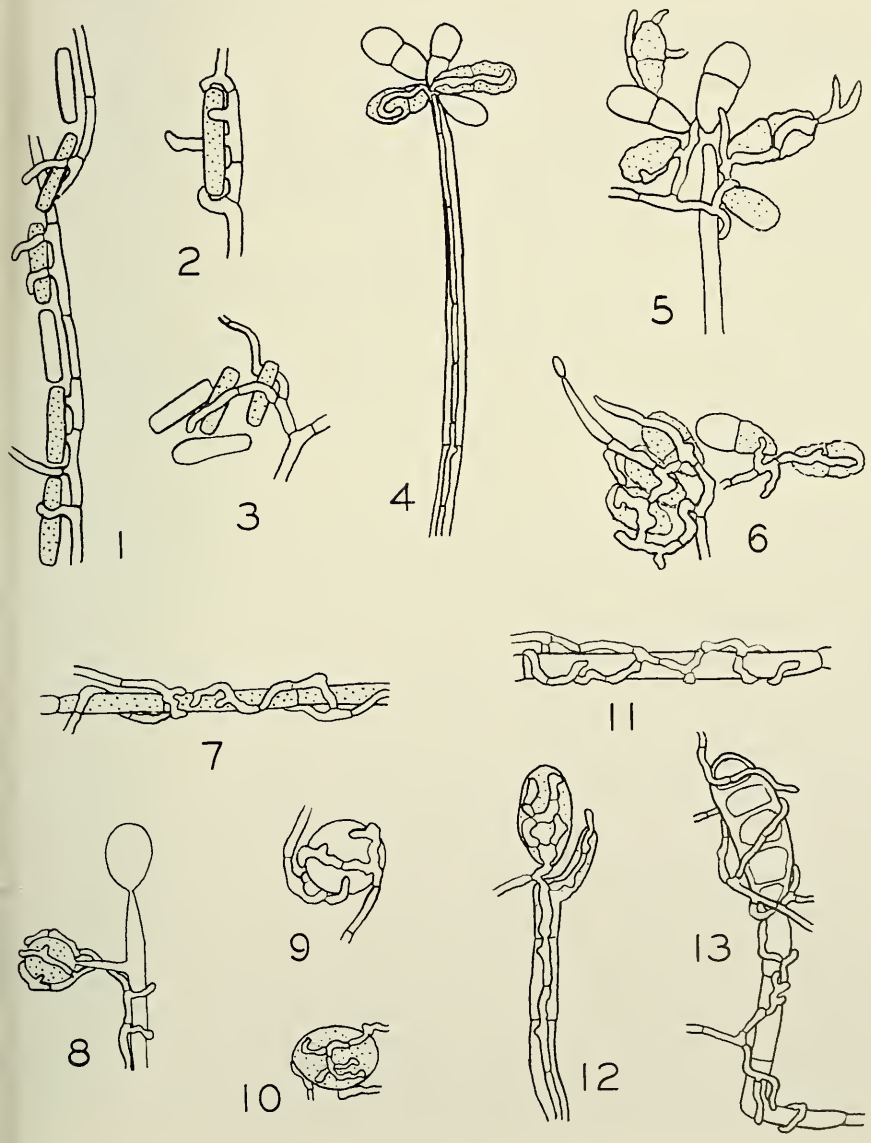
Basidiomycetes as mycoparasites: Recognition of certain basidiomycetes as destructive parasites of other fungi followed the observation that clamp-bearing mycelium of certain contaminating fungi overgrew cultures of some fungi. The first such isolate produced basidia and basidiospores. Single basidiospore cultures were obtained and found to be equally as virulent as the dicaryotic mycelium. The identity of this basidiomycete is still unknown.

The mode of parasitism was similar to that of *Gliocladium roseum,* in that contact between host and parasite always preceded killing of the host cells and that penetration was not common. The possibility

FIGURES 1-13. Gliocladium roseum parasitizing four host fungi. **FIGURES 1-3.** On conidia of Ceratocystis fimbriata. Note that only the spores contacted by the parasite are killed (shown by coarse stippling) without penetration. **FIGURES 4-6.** On Trichothecium roseum. Note that the contacted conidia are first killed and then may be penetrated by parasite hyphae. **FIGURE 6.** Killed spores showing disintegration of the walls. **FIGURES 7-10.** On Rhinotrichum macrospora. **FIGURE 7.** Parasite hyphae entwining and killing vegetative cells. **FIGURE 8.** Parasite hyphae climbing up conidiophore and surrounding an attached conidium. **FIGURE 9.** Detached conidium being surrounded by hyphae of the parasite. **FIGURE 10.** Detached conidium killed and penetrated by the parasite. **FIGURES 11-13.** On Helminthosporium sativum. **FIGURE 11.** Parasite hyphae entwining vegetative cells of the host. **FIGURE 12.** Conidiophore and immature conidium killed and penetrated by the parasite. **FIGURE 13.** Conidiophore and mature conidium surrounded by host hyphae but not killed.

that other common basidiomycetes may also be mycoparasites is being investigated. These studies have progressed only far enough to indicate that here is a fertile field for further study.

Miscellaneous destructive mycoparasites: Many miscellaneous fungi have been found growing on other fungi in nature, principally on the



fleshy fungi, and have been commonly described as fungicolous fungi. Tubaki (1955) has concluded that there are unknown growth substances in the mushrooms that favor growth of their parasites. This is an intriguing idea and may have merit but few mycologists have investigated this possibility.

Fusidium parasiticum was described by Backus and Stowell (1953) as causing a destructive disease of *Xylaria oxycanthae*. Parasitism occurred in culture as well as in nature.

The cytology of *Cicinnobolus cesati* as an internal parasite of mycelium and conidiophores of the powdery mildews has been adequately reported by Emmons (1930). This information should serve as a basis for a physiological study of the parasite.

Space does not permit listing or a discussion of the many observations of fungi that may belong to this group.

BALANCED OR BIOTROPHIC MYCOPARASITES

The term "biotrophic" is used to describe the parasite that normally obtains one or more of its essential nutrients from the living host cells (or hyphae). In this relationship a balance has developed in which the host continues to grow and provide a source of nutrients needed by the parasite.

To my knowledge, only seven species of balanced mycoparasites have received careful physiological study, and some of these have not been investigated intensively. These species, with the investigators, are: *Dispira cornuta* (Ayers, 1933, 1935); *Gonatorrhodiella highlei* (Ayers, 1941); *Piptocephalis virginiana* and *P. xenophila* (Berry and Barnett, 1957; Berry, 1958, 1959; Shigo *et al.*, 1961); *Calcarisporium parasiticum* (Barnett and Lilly, 1958); *Gonatobotrys simplex* (Whaley, 1961); *Gonatobtryum fuscum* (Shigo, 1960; Bishop, unpublished data).

In the following discussion emphasis will be placed on: (1) nutritional requirements, (2) the influence of host nutrition, (3) the degree of parasitism, and (4) axenic growth of the parasites. These are areas that have been emphasized in our studies aimed at learning more about the basic principles of parasitism.

Piptocephalis spp: The morphology and mode of parasitism are essentially the same for all species of *Piptocephalis* studied. The genus belongs to the Mucorales and is characterized by long, slender sporangiophores which give rise to one or more dichotomous systems of branches, each bearing a head of cylindrical sporangia which break up to form a series of rod-shaped spores. Parasitism is by means of slender branched haustoria. One of the first descriptions of the mode

of parasitism was made by Brefeld (1872), who studied *P. freseniana.* Benjamin (1959) has given the most recent review of the morphology and taxonomy of this genus. The host range of these species is limited to the Mucorales, with the exception of *P. xenophila,* which also parasitizes some species of ascomycetes and imperfect fungi (Dobbs and English, 1954).

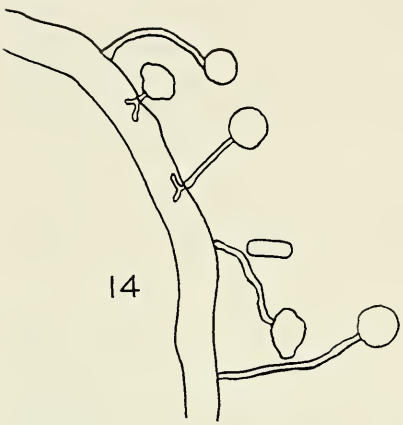
The studies of *Piptocephalis virginiana* have been confined to the only recorded isolate which was found growing on a species of *Mucor* near Morgantown, West Virginia, in 1953. It was described later as a new species by Leadbeater and Mercer (1957).

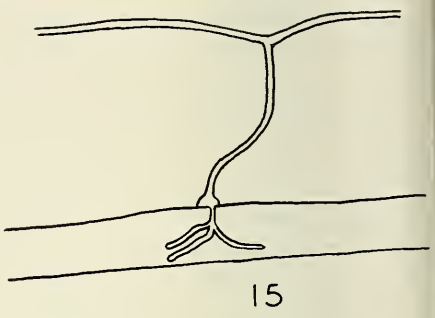
The spores of *P. virginiana* failed to germinate in distilled water or on water agar. Spore germination ranged from 10 to 90 per cent in extracts or media made from several natural products including yeast extract. Germination occurred on a glucose-asparagine-salts agar only in the presence of host fungi.

In the process of germination the spore swells and, usually within 24 hours, puts out one or more germ tubes that may continue to grow and branch, producing a very limited amount of mycelium before growth ceases. On a malt extract-yeast extract medium, about 10 per cent of the young mycelia produced upright dwarf sporangiophores, sometimes terminating in a head consisting of a few sporangia. When these spores were sown on the same medium, only a few germinated and produced short unbranched hyphae. This suggests that spores produced by the parasitic growth contain stored materials that are beneficial to further development. In the absence of a host this material becomes so diluted as to be ineffective. This compound is different from the spore germination factor contained in yeast extract.

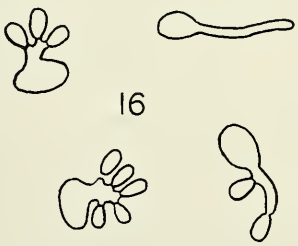
When the spores of *P. virginiana* germinated near the hyphae of a susceptible host, such as *Choanephora cucurbitarum,* the single germ tubes showed strong positive tropism toward the host (Figure 14). At the point of contact, an appressorium-like swelling was sometimes formed and penetration by an infection peg was followed by the formation of a branched haustorium (Figure 15).

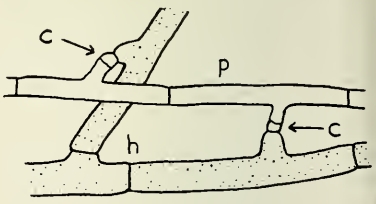
In incomplete host-range studies, 22 species of the Mucorales were susceptible, but of the 8 species of *Mortierella* tested, only *M. pusilla* was parasitized. Host species showing the highest degree of susceptibility were *Absidia orchidis, Syncephalastrum racemosum, Cunninghamella elegans, Mycotypha microspora,* and *Mortierella pusilla.* Both *Phycomyces blakesleeanus* and *Rhizopus nigricans* were highly resistant under most cultural conditions. *Thamnidium elegans* (low temperature isolate) was highly susceptible at 25° C and resistant at 15° and 20°

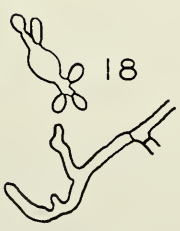


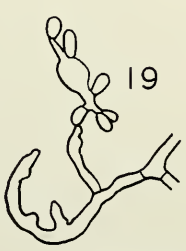


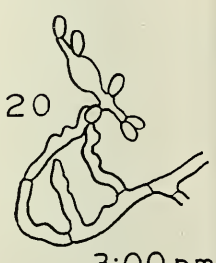
FIGURES 14-15. Piptocephalis virginiana. **FIGURE 14. Germ tubes showing positive chemotropism and penetrating the host hyphae. FIGURE 15. Slender branched haustorium in host hypha. FIGURES 14, 15 drawn from photographs (Berry and Barnett, 1957).**











FIGURES 16-20. Calcarisporium parasiticum. **FIGURE 16. Conidia germinating on glucose-yeast agar. FIGURE 17. Hyphae of parasite (p) and of host (h), showing the stubby contact branches and the small "buffer" cells. FIGURES 18-20. Branching and tropism of a single hyphal tip of the host near a germinated spore of the parasite, drawn at the times indicated.**

C, but *Helicostylum* sp. was more susceptible at 20° C than at 25° C. Both species showed a high degree of resistance near optimum temperatures for growth. Resistance to penetration of host hyphae was directly related to age and maturity in *Phycomyces blakesleeanus* and *Helicostylum* sp. Older portions of the hyphae of these species were not penetrated, suggesting mechanical resistance.

Piptocephalis virginiana was capable of making good growth on most hosts without causing noticeable harm or decrease in the growth rate. This parasite also grew well on host mycelium removed from the medium and washed with distilled water, showing that it can obtain all of its needed nutrients from its host. A search for specific compounds, such as amino acids, in the resistant and susceptible hosts failed to reveal any that might account for differences in host reaction.

One of the most important factors affecting the degree of parasitism was the nitrogen source in the host medium. Briefly, the growth of the parasite varied with the host species and the nitrogen source and was not correlated with the amount of host growth. Hosts were highly susceptible, generally, when grown on yeast extract, casein hydrolysate, and glutamic acid, whereas on phenylalanine, urea and ammonium sulfate they were only slightly susceptible (Table 1). Parasitism of *Thamnidium elegans* was only slightly affected by the nitrogen source. On asparagine medium the degree of parasitism was approximately equal on five hosts. As a specific example of host difference, *Mortierella pusilla* was immune on ammonium sulfate medium and moderately susceptible on phenylalanine medium, but the reverse was true for *Mucor ramannianus*.

Experiments to determine the effects of carbon-nitrogen ratios in the host medium showed that, in general, there was a direct correlation

TABLE 1. RELATIVE[a] GROWTH OF *Piptocephalis virginiana* ON HOSTS GROWN ON MEDIA CONTAINING GLUCOSE IN COMBINATION WITH NITROGEN SOURCES. TAKEN FROM BERRY (1959)

	Asparagine	Ammonium sulfate	Casein hydrolysate	Glutamic acid	Phenylalanine	Urea
Absidia orchidis	2	1	4	3	1	3
Mortierella pusilla	2	0	3	3	2	2
Mucor ramannianus ...	2	1	2	2	0	2
Rhizopus nigricans	2	0	1	1	2	0
Thamnidium elegans ..	2	2	3	2	2	2

[a]1 = slight, host highly resistant, to 4 = excellent, host highly susceptible.

between degree of parasitism (i.e., host susceptibility) and concentration of nitrogen furnished by yeast extract or casein hydrolysate, and an inverse correlation with the concentration of glucose. The best medium for growth of *P. virginiana* on its hosts was one containing 5 g glucose and 5 g yeast extract per liter. A 5-1 medium supported poor parasitism.

From the results obtained above on concentrations of host nutrients, it appeared likely that nitrogen starvation, rather than high sugar concentration, was directly responsible for poor growth of the parasite. A medium high in available carbon apparently resulted in extensive host growth and rapid depletion of nitrogen.

This theory was tested further using *Piptocephalis virginiana* and *Mortierella pusilla* in liquid media to determine whether the daily additions of a carbon source (glucose), with and without a nitrogen source (yeast extract), in sufficient quantities to maintain high carbon concentrations, would alter the degree of parasitism. The medium initially contained 5 g glucose and 5 g yeast extract per liter, and the residual glucose was determined daily. The parasite appeared on the fifth day and reached maximum growth on the 11th day in the control culture (Figure 21). In contrast, cultures receiving daily additions of

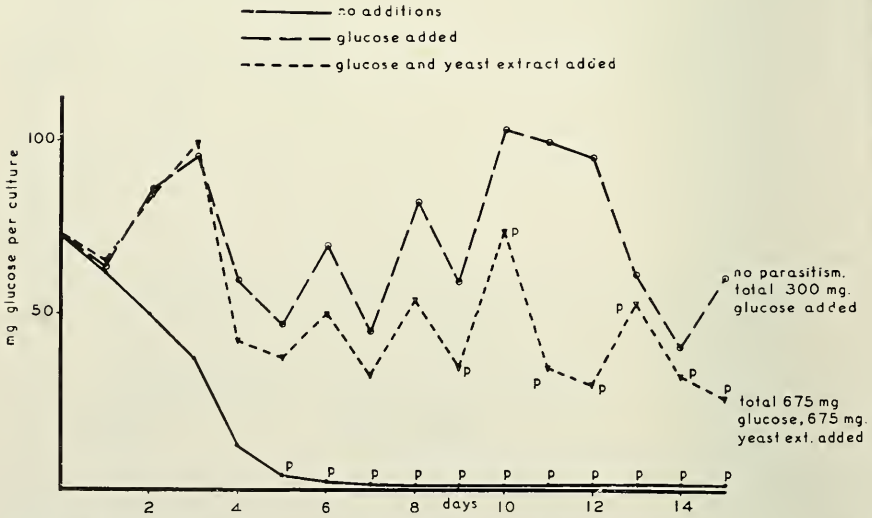


FIGURE 21. The effects of frequent additions of glucose and of equal amounts of glucose and yeast extract on the growth of Piptocephalis virginiana on Mortierella pusilla in liquid medium initially containing 5 g glucose and 5 g yeast extract per liter. P = presence of the parasite. From Shigo et al. (1961).

glucose alone (a total of 300 mg over a 14-day period) showed no growth of the parasite at the end of 15 days. In cultures receiving daily additions of equal amounts of glucose and yeast extract (a total of 675 mg of each over a 14-day period), the parasite appeared on the 9th day and reached maximum growth on the 15th day.

The conclusions are: (1) that rapid depletion of carbon source favored growth of the parasite, while high carbon concentration and nitrogen starvation inhibited parasitism, and (2) that high concentration of available nitrogen partially overcame inhibition by high carbon.

These results suggested that high susceptibility might be correlated with high concentrations of available nitrogen in the host mycelium. This was determined by growing *Mortierella pusilla* in liquid media varying in amounts and ratios of glucose and yeast extract and determining the percentage (by dry weight) of the soluble nitrogen (in cold trichoroacetic acid) in the mycelium. Briefly the cultures containing the highest concentration of glucose yielded the greatest weight of combined mycelium, showed the lowest degree of parasitism, and had lowest percentages of soluble nitrogen. The data at the end of 9 days are shown in Table 2, and were essentially the same at the end of 7 days. Similar results were obtained with *Penicillium frequentans,* a host of *Piptocephalis xenophila.*

TABLE 2. THE RELATION AND AMOUNTS OF GLUCOSE AND YEAST EXTRACT TO AMOUNTS OF TCA-SOLUBLE NITROGEN IN THE MYCELIUM OF *Mortierella pusilla;* PERCENTAGE OF SOLUBLE NITROGEN IS BASED ON DRY WEIGHT OF MYCELIUM. AGE OF CULTURES 9 DAYS. TAKEN FROM SHIGO *et al.* (1961)

Data/Culture	Initial medium: g glucose: g yeast extract			
	5:1	25:1	5:5	25:5
mg dry mycelium	87	226	126	353
% TCA-nitrogen	0.68	0.32	1.61	0.55
mg residual nitrogen	1.38	1.08	5.92	4.62
mg residual glucose	3	332	4	100
rank of parasitism	poor	none	excellent	trace

Further studies indicated that the addition of an excess of the essential micro elements, Mn, Fe, and Zn, up to four times the usual amounts added to media, increased the degree of parasitism of *P. xenophila* on *Penicillium frequentans* in liquid media. Manganese alone was nearly as effective as the three micro elements together.

Further evidence of the favorable effects of high concentrations of micro elements was discovered in studies on *Gonatobotryum fuscum* and will be discussed later.

Dispira cornuta: This parasite is similar to *Piptocephalis* spp. in host range and general mode of parasitism. In contrast to the results discussed with *Piptocephalis* spp., Ayers (1933) succeeded in obtaining continued axenic growth and heavy sporulation of *D. cornuta* on special media high in protein or natural nitrogens compounds. This was later confirmed by Benjamin (1959).

These results are significant because they represent the first successful attempt to obtain growth of an "obligate" mycoparasite in culture free from the living host. It suggests that the same required nutrients are present in the living host and in the high-nitrogen natural products, but not present in sufficient quantities in common laboratory media. Ayers also pointed out that the use of agar was unsatisfactory for the study of some parasites because of its inhibitory action. Perhaps the more highly purified agar now available may not have this effect. *Dispira simplex* Benjamin (Benjamin 1959, 1961) is also of interest because it parasitizes species of *Chaetomium,* but not species of Mucorales.

Calcarisporium parasiticum: The three known species of *Calcarisporium* have been found consistently associated with other fungi and are all considered mycoparasites. *C. pallida,* described by Tubaki (1955), has received little study and *C. arbuscula* is found on fleshy fungi in nature (Watson, 1955), but can be cultivated easily on common synthetic media. *C. parasiticum* is known only from cultures growing on its hosts isolated in West Virginia (Barnett and Lilly, 1958; Barnett, 1958).

The known host range of *C. parasiticum* is limited to the genus *Physalospora* and related fungi. Susceptible hosts include *Physalospora obtusa* (Figure 22), *P. ilicis, P. glandicola, Botryosphaeria ribis, Guignardia bidwellii, Diplodia pinea, Dothiorella* sp., and *Coniothyrium* sp. Five isolates of *P. rhodina* (*Diplodia natalensis*) and one isolate each of five additional species of *Physalospora* were immune.

Calcarisporium parasiticum has been of special interest because of the several physiological adaptations to its specific mode of parasitism and growth. The conidia failed to germinate in distilled water or on water agar, but a high percentage germinated within 48 hours on a glucose-yeast extract agar. The germination factor was present in extracts of host fungus mycelium, but several synthetic media containing amino acids failed to induce germination.

FIGURE 22. Calcarisporium parasiticum, **the white fungus, overgrowing the dark mycelium of the host,** Physalospora obtusa.

The usual method of germination is by the production of short germ tubes bearing a few secondary spores (Figure 16). No further development of the parasite occurs in the absence of a susceptible host. *C. parasiticum* normally does not produce long germ tubes on a medium favorable for germination, and has adopted a more or less unique way of meeting its host. Host hyphae growing among scattered germinating spores of the parasite are stimulated to send out lateral branches which grow directly toward the parasite, or the main hypha may curve from its original direction toward the spores of the parasite (Figures 18-20). The stimulus is presumably a chemical substance secreted by the germinating parasite spores. It is of interest that *Physalospora rhodina,* an immune species, also showed positive tropism as readily as did *P. obtusa.* Most of the immune species did not react chemotropically.

Actual contact is often brought about by means of short stubby branches of host and parasite, resulting in a very small area of contact. A small cell is formed at the tip of the contact branch of the parasite (Figure 17). This tiny "buffer" cell, while not always visible, is believed to occur in all or nearly all contacts with susceptible hosts, but it was not seen when contact was made with immune species.

The function of the buffer cell is not clear but it appears to be related to the nutrition of the parasite. It possibly functions as a site of synthesis of enzymes or other compounds that act to increase the permeability of the host cell membrane at the point of contact. Further evidence to support this idea is the fact that within a period of 3 or 4 hours following contact, the parasite begins to grow. Branching and sporulation frequently occurs within 24 hours. Such a rapid development could result only following rapid absorption of required nutrients from the host through this single point of contact. The parasitized host hyphae are soon severely inhibited or cease to grow. This suggests that the same compound is required for growth by both host and parasite.

The degree of parasitism varied with the host species, the isolate of the parasite, and with the nitrogen source in the host medium. The ranking of the degree of parasitism, based on amount of growth of the parasite on agar cultures of the hosts, is only an estimate and is subject to much error. A more accurate objective measure of the inhibitory effect on host growth was used for cultures grown in liquid media. In preliminary experiments it was found that the dry mycelial weights of mixed cultures of the susceptible host and parasite were always lower than those of host mycelium alone under the same conditions.

Four isolates of the parasite were much alike in their effect on *Physalospora obtusa*, reducing the host growth by 25 to 50 per cent by the eighth day (Figure 23). The actual reduction in host weight was much greater, since much of the weight of the mixed cultures was that of the parasite. Nitrogen source was an important factor, as shown by comparison of growth on glucose-casein hydrolysate and on glucose-potassium nitrate medium. Early depression of the host in particular was much more severe on nitrate even though the host alone grew slower on this medium.

Three isolates of *P. obtusa* varied considerably in susceptibility. Isolates 1130 and 1166 had nearly identical growth curves when grown alone on nitrate medium. However, isolate 1130 was much more susceptible to *C. parasiticum* than was 1166 (Figure 24).

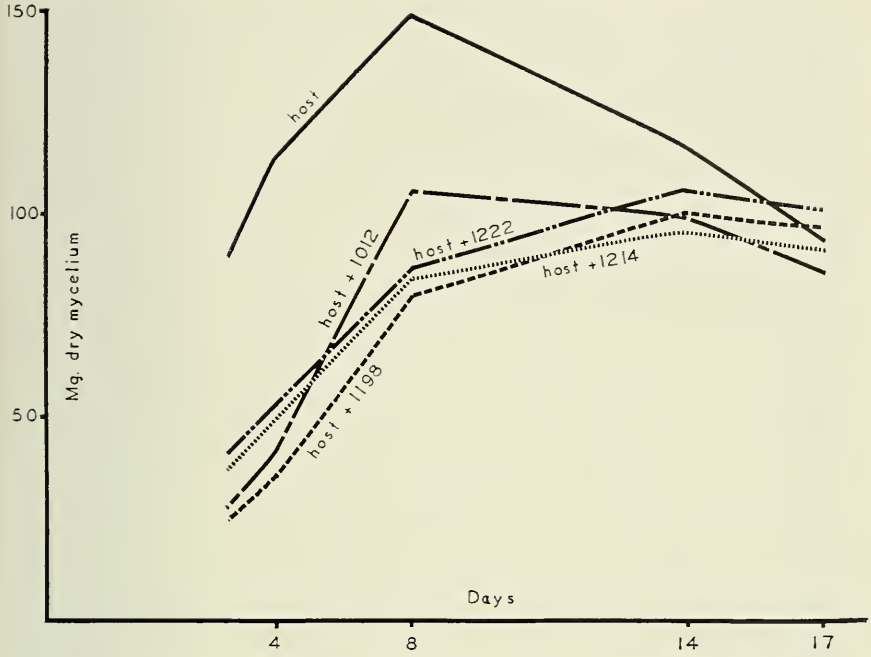


FIGURE 23. Growth of the host, Physalospora obtusa, **alone and in combination with each of four isolates of the parasite,** Calcarisporium parasiticum, **in liquid glucose-casein hydrolysate medium.**

The effects of the carbon-nitrogen ratio was also noted on *C. parasiticum,* with the host species showing a high degree of variation in susceptibility.

Physalospora obtusa, a highly susceptible host, allowed very good growth of the parasite regardless of the carbon-nitrogen ratio (Table 3). *P. ilicus* was highly resistant on 20-1 and 10-1 media, but highly susceptible on 3-4 and 3-8 media. Growth of the parasite on *Coniothyrium* sp. was light on the high nitrogen media and no growth occurred on the high carbon media.

Up to this point in the study of *Calcarisporium parasiticum* the parasite grew only in the presence of a living susceptible host. But every host-parasite relationship presents a challenge to break this dependence on the living host by the addition to the media of the nutrients required by the parasite. The most logical starting point in this study would be the addition of water extracts obtained from the host to the medium.

In contrast to the results of earlier attempts to culture *Piptocephalis virginiana* in the absence of a host, slow continuous axenic growth of *C. parasiticum* was obtained on a glucose-yeast extract medium to

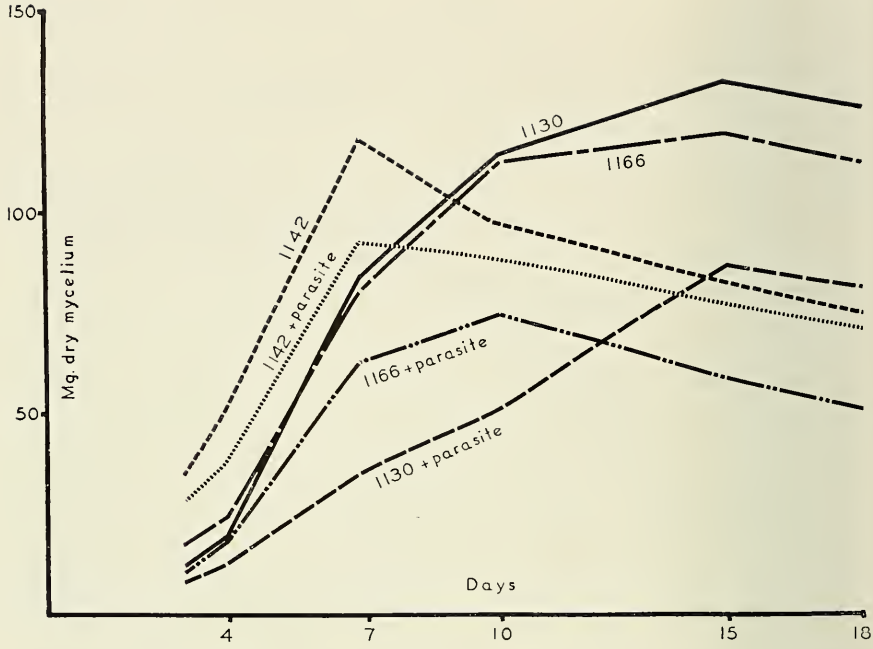


FIGURE 24. Comparison of growth of three isolates of Physalospora obtusa **alone and with** Calcarisporium parasiticum **in a glucose-potassium nitrate medium.**

TABLE 3. ESTIMATED RELATIVE GROWTH OF *Calcarisporium parasiticum* ON THREE HOSTS CULTURED ON FOUR AGAR MEDIA DIFFERING IN CONCENTRATIONS OF GLUCOSE AND CASAMINO ACIDS. GROWTH IS RECORDED IN FIVE RANKS: 0 (NONE), 1 (SLIGHT; HOST HIGHLY RESISTANT) TO 4 (EXCELLENT; HOST HIGHLY SUSCEPTIBLE). FROM BARNETT AND LILLY (1958)

Host and time	20 Gl.-1 CA	10 Gl.-1 CA	3 Gl.-4 CA	3 Gl.-8 CA
Physalospora obtusa				
5 days	2	2	2	3
10 days	4	4	4	4
P. ilicis				
5 days	0	0	1	1
10 days	1	2	4	4
14 days	1	2	4	4
Coniothyrium sp.				
5 days	0	0	0	0
10 days	0	0	1	1
14 days	0	0	1	2

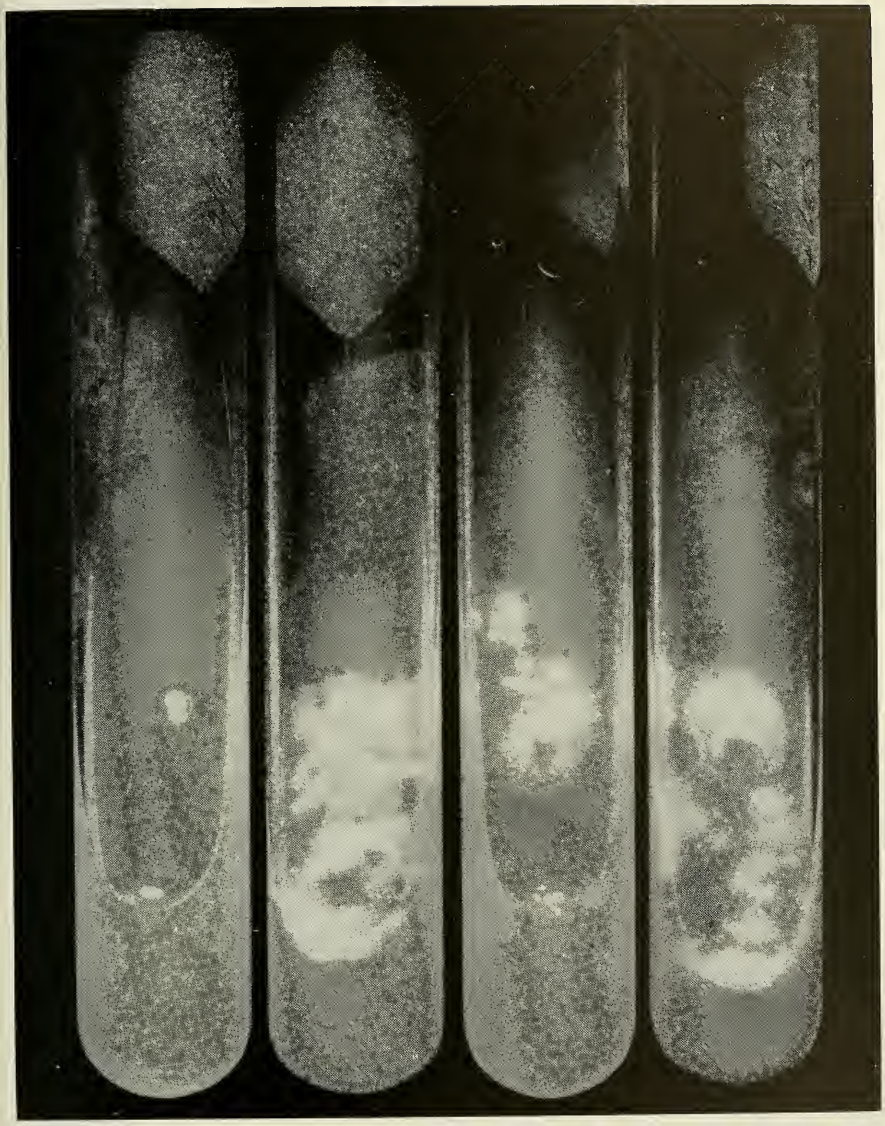


FIGURE 25. Calcarisporium parasiticum. The effects of adding host extracts on growth of the parasite on glucose-yeast extract agar medium. From left to right: no extract added, extracts of Physalospora obtusa (a highly susceptible host); of P. ilicis (a moderately susceptible species); P. rhodina (an immune species).

which a water extract of *Physalospora obtusa* had been added. Later a similar extract from *P. rhodina,* a species immune to *C. parasiticum,* was found to be equally effective in stimulating axenic growth of this parasite (Figure 25). Further studies on the occurrence, preparation,

and partial purification of the active principle in the extract will be discussed later in reference to parasitism by *Gonatobotrys simplex.*

Additional studies on the nutritional requirements of *C. parasiticum* showed that no growth was made on media containing only a single amino acid, except glutamic acid. Thus, this parasite is one of the few fungi isolated from nature that has a deficiency for glutamic acid. Although growth of *C. parasiticum* occurs on a semisynthetic medium containing glutamic acid and a small amount of host extract, the sporulation under these conditions is not normal. The conidiophores are short, simple or sparingly branched and do not show the typical verticillate branching. It is possible that additional nutrients or a different balance of nutrients are required for normal sporulation.

Gonatobotrys simplex: This fungus has been isolated from nature only in mixed culture with other fungi, principally *Alternaria* or related fungi. Drechsler (1950) studied one isolate and concluded that it was not a nematode trapper and not a mycoparasite. However, the one isolate studied in our laboratory was shown to be parasitic on *Alternaria* spp. and *Cladosporium* sp. The mode of parasitism and the nutrients required from the hosts were very similar to those of *Calcarisporium parasiticum.*

Conidia of *G. simplex* failed to germinate on water agar or synthetic agar media. On glucose-yeast extract agar the spores produced a few bud-like cells (Figure 27), but no further development occurred in the absence of a susceptible host fungus. Hyphae of *Alternaria tenuis* were attracted to the germinating spores of the parasite (Figure 28) much in the same manner as hosts of *C. parasiticum* showed chemotropism toward germinating spores of this parasite. Contact between host and parasite was made by short stubby branches, but no buffer cell was produced and there was no penetration of the host cells (Figures 29, 30).

The addition of the same fungus extracts effective in producing axenic growth of *C. parasiticum* likewise resulted in good growth and in normal sporulation of *G. simplex* on a 5-5 glucose-yeast medium (Figure 26). The water soluble extract was partially purified by boiling with Norit (activated charcoal), eluted with 10 per cent aqueous pyridine, evaporated to dryness, and redissolved in distilled water. The active principle (the growth factor) was found to be heat stable (withstanding autoclaving for several hours), soluble in water, 95 per cent ethyl alcohol, and acetone, and insoluble in di-ethyl ether, chloroform, and petroleum ether. The partially purified compound was effective in concentrations as low as 1 ppm. Further tests showed

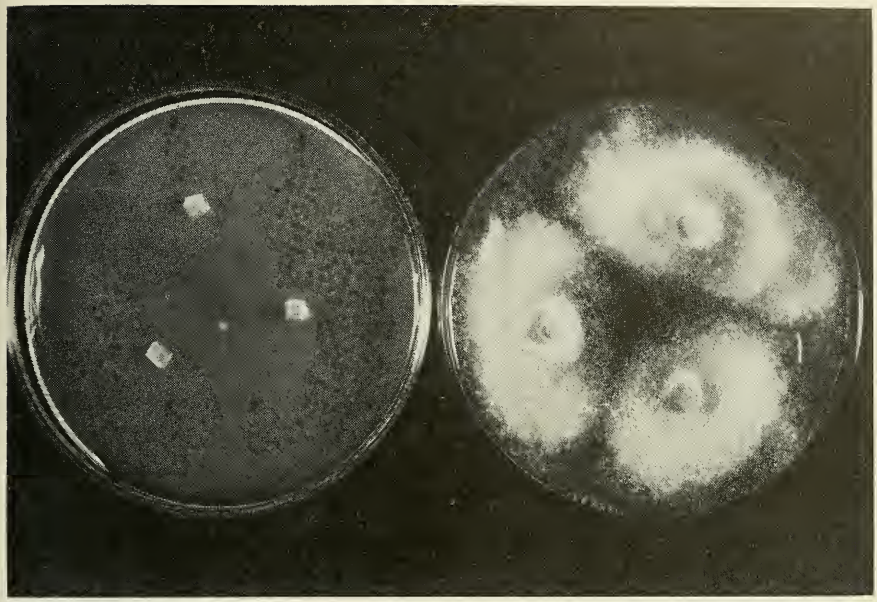


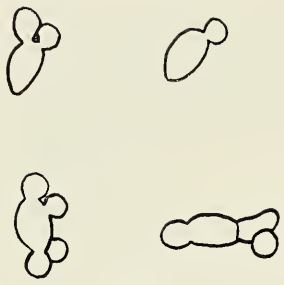
FIGURE 26. Gonatobotrys simplex. **The effects of adding one drop of the partially purified growth factor on the axenic growth of the parasite. The plate on the left received no growth factor. The medium is glucose-yeast extract agar. From Whaley (1961).**

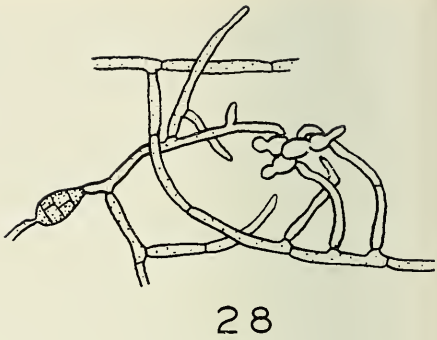
that the growth factor could be recovered from a band on chromatographic paper. The need for this nutrient by these two mycoparasites was not replaced by any of the B vitamins, nor by any of the many chemicals tested. It appeared to be a new growth factor and was tentatively called "mycotrophein" (fungus nutrient), by Whaley (1961).

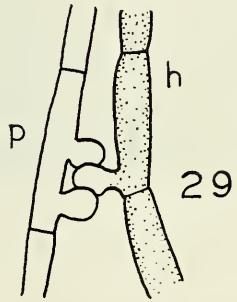
This growth factor was found to be present in the mycelium of many fungi, hosts and non-hosts alike. Of the total of more than 100 species tested for its occurrence, most of the ascomycetes and imperfects were positive as were some basidiomycetes, but all of the phycomycetes tested were negative. It was not found in any higher plants or animal products tested.

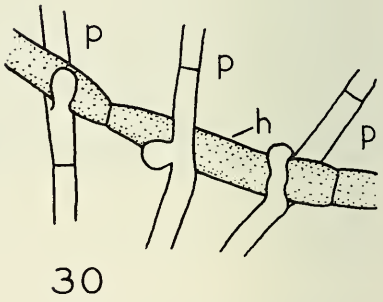
We may conclude that this compound is a common and probably essential metabolite of many fungi, which synthesize it and normally retain it within their living cells. Most fungi do not excrete it into the surrounding medium, unless the permeability of the cell membrane is altered.

One compound reported to alter permeability is dodine, or Cyprex (Brown and Sisler, 1960). There is some evidence that a drop of dodine solution added to an agar plate near a colony of *Arthrobotrys musiformis* brought about an excretion of the growth factor, for spores of *G. simplex* in the same culture germinated and produced mycelium.

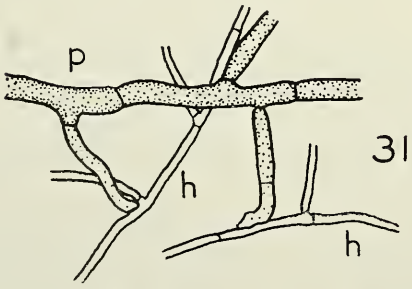


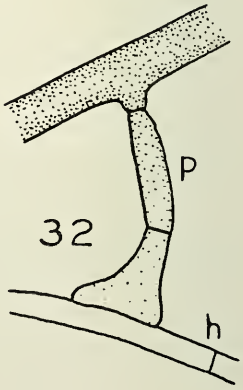






FIGURES 27-30. Gonatobotrys simplex. **FIGURE 27. Conidia germinating by budding on glucose-yeast extract agar. FIGURE 28. Tropism of hyphae of Alternaria tenuis toward germinating spore of G. simplex. FIGURES 29, 30. Hyphae of G. simplex (P) and A. tenuis (h) showing globose to elongate contact branches.**





FIGURES 31, 32. Gonatobotryum fuscum (P) and Graphium sp. (h), showing elongated absorptive branches and the contact points with the host. Drawn from photographs by Shigo (1960).

Some non-host fungi and bacteria excrete the growth factor into the substratum. A colony of *Zygosporium masonii* appeared as a contaminant near the edge of a plate inoculated at the center with *G. simplex*. An antibiotic prohibited growth within a wide zone around *Z. masonii*, but *G. simplex* grew and sporulated well at the edge of this zone (Whaley and Barnett, 1961).

Still further nutritional studies showed that *G. simplex* utilized glucose, fructose, galactose, and mannose, but not sorbose or disaccharides. Nitrogen sources containing mixtures of amino acids were utilized well, but little growth occurred on any single amino acid.

Gonatobotryum fuscum: This genus is the dark counterpart of *Gonatobotrys*, both producing dry conidia on denticles on swollen cells of the tall conidiophores. *G. fuscum* was first reported as a mycoparasite in England (Vincent, 1953), but no physiological study was made at that time. The few isolates of this species were collected in West Virginia growing on species of *Graphium* or *Ceratocystis*.

Gonatobotryum fuscum was found to be a balanced mycoparasite, making contact with but not penetrating its host (Figure 31, 32), much like *C. parasiticum* and *G. simplex*. Unlike these mycoparasites, it failed to grow axenically when the growth factor mycotrophein was added to a glucose-yeast extract medium. The known host range is limited to nine species of *Ceratocystis*, two species of *Graphium*, and one of *Leptographium*.

G. fuscum made good growth on *Graphium* sp. in continuous total total darkness, growing well beyond the host colony, but parasitism was poor in continuous light. Synnema formation by *Graphium* sp. was suppressed in darkness. Similarly, the growth of the parasite disrupted synnema formation in alternate light and darkness. These results suggest a close relationship between metabolites required for synnema formation by the host and for the growth of the parasite.

In older cultures of this parasite on *Graphium* sp., the hyphae of the latter continued to grow aerially and to coil around and often penetrate the large conidiophores of *G. fuscum*, causing their collapse. This is believed to be an example of reversal of parasitism, but the details of this relationship will require more study.

In contrast to the balanced parasites discussed above, *G. fuscum* appeared to be favored generally by a host medium containing a relatively high carbon-nitrogen ratio.

It was later discovered that the addition of micro elements, Mn, Zn, Fe, and Ca, in excess of the amounts needed for maximum host growth, favored development of the parasite on some media that

otherwise were unsatisfactory for the parasite. The addition of excess micro elements to some media also greatly increased susceptibility of the host. Manganese in combination with Fe or Ca was nearly as effective as all four elements.

Recent unpublished information (Bishop) has shown that good growth of *Gonatobotryum fuscum* on a susceptible host is related to fatty acid synthesis by the host. Based on this information, additional attempts were made to obtain axenic growth of this parasite. Partial success was attained in a special medium to which extracts of host mycelium were added.

DISCUSSION

It would be dangerous to generalize about the physiology of mycoparasitism, because so few of the available species have been studied. Enough facts have been learned, however, that it would be helpful to summarize them and to look for relationships and developing patterns or principles.

The destructive mycoparasite, in order to compete successfully with saprophytic species, must have the ability to produce enzymes or toxins that inhibit or kill and destroy associated fungi, their hosts, and utilize the nutrients released. The true destructive mycoparasite is well exemplified by *Gliocladium roseum,* which has a broad host range and the ability to completely destroy established colonies of some host species. *Rhizoctonia solani* has a narrower host range and less ability to destroy host colonies.

It would seem that future studies of destructive mycoparasitism should be aimed first, at a better understanding of mode of parasitism of a number of additional host-parasite combinations and second, at determining the nature and identity of the active toxic principle.

In contrast to the destructive mycoparasite, the strength of the balanced or biotrophic mycoparasite lies in its ability to contact a suitable host and obtain from it specific elaborated nutrients for which the parasite is deficient, without disrupting the ability of the host to produce these compounds. Following the establishment of a favorable nutritional relationship with its host, the development of the parasite may be influenced by many factors.

The spores *Gonatobotryum fuscum* germinate in distilled water, but spores of *Piptocephalis* spp., *Calcarisporium parasiticum,* and *Gonatobotrys simplex* require specific nutritional factors for germination. It appears that these nutrients are furnished by the host fungi, but no extensive study of them has been made.

Contact between parasite and host hyphae may not be due entirely to chance. Germ tubes of *Piptocephalis virginiana* often show positive chemotropic growth toward nearby host hyphae. *Calcarisporium parasiticum* and *Gonatobotrys simplex*, which do not produce long germ tubes, except in the presence of the specific growth factor, have a unique method of making contact with the host. The host hyphae or branches show decided positive chemotropism toward the germinating spores of the parasite.

Establishment of a food relationship is believed to occur immediately after penetration or close contact with susceptible host species. The host must not only contain the nutrients needed by the parasite, but the parasite must be able to absorb the nutrients from the living host cells, probably by altering the permeability of the host cell membranes.

Estimation of the amount of parasite growth by the usual 0-4 rating gives no real measure of the effects of the parasite on its host. Such a measure of inhibition of growth can be obtained on the cultures grown in liquid media. *Piptocephalis* spp. caused little or no depression of dry weights of most hosts, but depression by *Calcarisporium parasiticum* was often as much as 50 per cent. The actual inhibition of host growth is greater than dry weights indicate, because the host and parasite mycelia growing in the same culture must be weighed together.

The two principal environmental factors that affect the degree of parasitism are temperature and light, and these appear to affect hosts in different ways. Butler (1957), and Shigo (1960) have reported that continuous darkness favors the parasites they studied. Parasitism by *Gonatobotrys simplex* (Whaley, 1961) and *Piptocephalis* spp. is not affected by light or darkness. The disruption of synnema formation in *Graphium* sp. by either darkness or by growth of *Gonatobotryum fuscum* may mean that both host and parasite compete for an intermediate metabolite required for production of synnemata.

The type of nitrogen source in the medium was one of the most important factors determining the degree of parasitism. In general the best nitrogen sources were those containing a mixture of amino acids. The effects of single amino acids and inorganic nitrogen compounds varied with the parasite and the host, although there was no direct correlation between good growth of host and degree of susceptibility. All evidence points toward the conclusion that the effects of nitrogen source and concentration are primarily on the metabolism of the host, altering its susceptibility, rather than affecting the parasite directly.

The degree of host susceptibility is greatly affected by the ratio of carbon to nitrogen, as described by Shigo *et al.* (1960) for *Piptocephalis* spp. It is assumed that these mycoparasites find a plentiful supply of soluble nitrogen in the host hyphae favorable for growth, or for production of enzymes needed in their parasitic activities. The response of *Calcarisporium parasiticum* to the carbon-nitrogen ratio in the medium varied with the susceptibility of the host species, the highly susceptible species being least affected by changes in concentrations or ratios.

The response of *Gonatobotryum fuscum* to concentrations of carbon in the host medium differed from that of other parasites tested. A relatively high concentration of carbon favored growth of the parasite. The meaning of this is not yet clear, but it is believed to be related to lipid synthesis in the host (Bishop, unpublished data).

The effects of high concentrations of micro elements first noted by Shigo (1960) and later studied by Bishop, appeared to be on the metabolism of the hosts, and again may be related to lipid synthesis.

The discovery of a water-soluble, heat stable substance extracted from host fungi and required for axenic growth of two mycoparasites, *Calcarisporium parasiticum* and *Gonatobotrys simplex*, gives us greater insight into their basic nutritional requirements. Partial purification of this substance and demonstration of its effectiveness in extremely small amounts suggest that the active compound is in the nature of a growth factor, differing from known B vitamins. The presence of the growth factor in many non-host fungi, as well as hosts, indicates that it is a normal metabolite required by a large number of organisms, but the requirement can be demonstrated only in those unable to synthesize it.

The conclusion follows that the successful establishment of a parasitic relationship between these biotrophic mycoparasites and their potential hosts is determined by the ability of the parasite to absorb the specific required nutrients from the living cells of the potential host.

In summary, there have been five points that stand out in the work on mycoparasitism in our laboratory during the past seven years. Briefly these are:

1. The demonstration that high susceptibility of host fungi to certain biotrophic parasites is directly correlated with high concentrations of soluble nitrogen in the host.

2. The extraction and partial purification of a new growth factor from both host and non-host fungi and the demonstrated axenic growth of two biotrophic mycoparasites in the presence of this nutrient.

3. The finding that high concentrations of micro elements, in excess of that needed for maximum host growth, increase growth of certain parasites and that this increased growth probably acts on the metabolism of the host to make it more susceptible.

4. The discovery that degree of susceptibility to specific mycoparasites may be related to the presence or synthesis of certain lipid compounds.

5. The recognition that many common saprophytic fungi (including some basidiomycetes) may also be destructive mycoparasites capable of completely destroying established colonies of some host fungi under favorable conditions.

The future of these investigations into the physiology of mycoparasitism depends to a large extent upon interested personnel, well-trained in biochemistry and techniques of fungus physiology. Certainly there is no shortage of available mycoparasites for further study. It is hoped that in future investigations the "guinea pigs" of the fungus world will contribute their fair share to our knowledge of basic principles of parasitism, and that these principles may help us better to understand the physiology of plant disease.

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The Physiology of Wilt Diseases

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INTRODUCTION—Fifty years ago, West Virginia University began its studies in plant pathology. The subject has grown vastly in the intervening years. Many of the significant discoveries in plant pathology have come from the scientists here. For 50 years, the people of West Virginia have benefited directly through lowered crop losses from plant disease and improvements in the quality and quantity of food and fiber. Through the years, the work at West Virginia on physiology of fungi and physiology of parasitism has been outstanding, and the names of Barnett, Leach, Leonian, Lilly, and True have become intimately associated with the development of the subject as a whole. It is a privilege and a genuine pleasure to participate, with the Potomac Division of the American Phytopathological Society, in celebrating 50 years of plant pathology at West Virginia University.

Physiological plant pathology was in its infancy 50 years ago. Volume 1 of *Phytopathology* contained less than half a dozen articles touching on the subject. Today, the physiology of the diseased plant and host-pathogen interactions are in the forefront. In a recent issue of *Phytopathology,* now in its 52nd year, 15 of the 20 articles dealt with physiological aspects of plant diseases. The physiological approach and a deeper understanding of the nature of plant disease are a part of the current trend in our field.

The wilt diseases began to be considered from a physiological point of view just about 50 years ago. In 1913, Hutchinson (23), studying a wilt disease of tobacco, proposed that toxic substances, produced in wilt-infected plants, cause damage to the host at a distance from the pathogen itself. Since that time the physiology and biochemistry of wilt disease have been intensively investigated. To attempt to discuss all facets of the subject is superfluous, in view of the quality and number of existing reviews on wilt diseases and their physiology (4, 11, 12, 17, 18, 22, 32, 37, 44, 49, 50, 51). Instead, I shall present some old and some new views, hoping to stimulate thought and research that will lead to a better understanding of these diseases.

The role of fusaric acid.—Fusaric acid is now believed to play a role in a number of wilt diseases. First isolated by Yabuta *et al.* (53) from *Fusarium heterosporum,* it has since been demonstrated as a product of a number of forma species of *F. oxysporum,* and of *F. solani, F. lateritium, F. moniliforme, Nectria cinnabarina,* and *Gibberella fujikuroi* (32). Initially isolated from culture filtrates of *F. oxysporum lycopersici,* fusaric acid was suggested as the active principle in pathogenesis of tomato wilt (19). Later it was shown to be present in infected plants (25, 26, 30). Subsequently Kalyanasundaram (24) demonstrated the formation of fusaric acid in the rhizosphere of the tomato plant.

Chemically, fusaric acid is 5-n-butyl picolinic acid. Preparations also contain dehydrofusaric acid, 5-n-butenyl picolinic acid (18). Both of these compounds chelate metals and the iron chelate is more toxic than is fusaric acid itself (29). Data given by Gaumann (18) indicate a half life in the tomato shoot of about 12.25 hours, which suggests that a high rate of synthesis in infected plants is necessary if a toxic titer is to be produced and maintained.

In the diseased plant or in plants treated with fusaric acid, two other compounds are found, 3-n-butyl pyridine which, though incapable of chelation, is 100 times as toxic as fusaric acid in altering permeability of cells to water, and the N-methyl amide of 5-n-butyl picolinic acid, having a quaternary ammonium ion, which is nontoxic (18, 28). The latter compound is an end product of detoxifying action of the plant.

In pathogenesis, the chelating action of fusaric acid may rob the host cell of iron and other metabolicially essential metals (18). That iron chelation may be important was suggested by Subramanian (43), who pretreated cotton shoots with 8-quinolinol and protected them from the toxic action of culture filtrates containing fusaric acid. 8-Quinolinol also acted as a chemotherapeutant on *Fusarium*-infected cotton plants. Lakshminarayanan (29) found that resistant varieties of cotton contain considerably higher amounts of cystine than susceptible ones do, and protected susceptible cotton shoots against injury by the fusaric acid-iron complex by allowing them to absorb cystine. But Malini (34) has shown by spectrophotometric and chromatographic methods that cystine does not chelate iron *in vitro.* Thus, if cystine plays a role in the resistance of cotton to wilt, it does not act by preventing fusaric acid from chelating iron. Fusaric acid is a weak chelating agent and whether it can bind more than ionic iron, whether, in fact, it can induce a physiological iron deficiency in the plant is problematical (32).

That fusaric acid, dehydrofusaric acid, and 3-n-butyl pyridine have specific effects on the permeability of parenchymatous cells to water is well known (17, 18). This effect cannot be wholly ascribed to chelating activity because 3-n-butyl pyridine is incapable of chelation yet is more active than fusaric acid.

Respiratory disturbance occurs in diseased plants. Nishimura (35), studying *Fusarium*-infected watermelon plants, found the content of ascorbic acid and gluathione to be increased about 1.5-fold and polyphenol was increased about fivefold, relatively to healthy plants. Some workers have ascribed this effect to the action of fusaric acid (18). Thus Bossi (3) has shown that fusaric acid competitively inhibits polyphenoloxidase, whereas it inhibits catalase and ascorbic acid oxidase only at high concentrations. Other enzymes are also affected.

We may conclude that fusaric acid has many different effects on plant cuttings and its importance *in vivo* depends upon how high the titer rises and where these effective titers occur. Because fusaric acid is capable of damaging cells in so many ways, its effects cannot be expected to be displayed in a simple way.

The role of growth factors.—A number of the wilt diseases display a formative symptom early in the syndrome. Leaves may be unilaterally distorted in *Verticillium* wilt of mint, they may show epinasty in tomato wilt, and the shepherd's crook symptom in very early stages of Dutch elm disease is a familiar one.

These effects are apparenty ascribable to the effects of compounds having growth-stimulating activity. In *Fusarium* wilt of tomato, the pathogen is known to produce ethylene *in vitro* and infected plants liberate sufficient ethylene to induce epinasty in healthy plants confined with them (15). Fusaric acid causes a downward bending of petioles of treated tomato cuttings, reminiscent of ethylene-induced epinasty (17). In *Verticillium*-infected tomato plants Pegg and Selman (36) have demonstrated auxin levels approximately twice those of normal plants.

Ethylene, excess of auxin, and fusaric acid can all produce side effects upon the infected plant, including effects upon growth habit and respiration among others. The magnitude of these effects in damaging the plant and reducing its productivity is, as yet, not fully appreciated.

The role of dysfunctional xylem in wilting.—In wilt diseases, the water economy of the plant becomes bankrupt. *Verticillium*- and *Fusarium*-infected tomato plants lose more water than they gain over a period of time, despite eventually reduced transpirational losses (14, 31, 39, 45). This shortage does not result from inability of the root

to absorb water (31). As infection progresses, the vascular system offers increasing resistance to flow of water. As water becomes ever more limiting, stomates become permanently closed (14). Leaves wilt and then die.

In young plants the transpiration stream moves through the conductive xylem of the primary vascular skeleton, which traverses the stem and petioles. In the tomato plant, this skeleton has been accurately described (1, 31). The cross section of the tomato stem ordinarily shows 3 large vascular bundles and 3 small ones, alternating with them. The large bundles form a network, connected through the vascular arches over each node. The maximum distance in the stem without a network connection for a large bundle is 2 nodes and the minimum is 1 node. The small bundles arise above a node, traverse 3 nodes through the stem without connection to other bundles, and form the central bundle through the petiole. Lateral bundles in the petiole arise at the node of origin of the leaf and also traverse the petiole without branching.

The individual bundles contain conductive elements in the form of tracheids and vessels. Vessels form more or less continuous capillaries with irregular perforations at intervals where vessel segments abut and especially where vessels end. Along the walls of the vessels and tracheids are many pits of varying size and shape, depending upon the age of the plant when the vessel was produced. Both vessels and tracheids are leaky tubes and perforations in pit membranes provide a continuum from one cell to another laterally. An insight into the manner of sap flow in angiosperms can be obtained from the studies of Scholander, *et al.* (40, 41), who have stressed the dynamic function of pit membranes and endings of vessels in preventing movement of gases and other materials.

The flow of the transpiration stream through conductive xylem is not turbulent but is laminar (46). This suggests that energy losses in water movement through xylem are small. When water flows through a capillary system in laminar fashion, it follows Poiseuille's law. Ludwig (31) has shown that water movement in the tomato stem through vascular bundles follows Poiseuille's law. In addition to this, the flow of water through pit membranes where openings are much smaller should also obey this law. Elford (16) made use of this principle in devising methods for calibrating the filtration properties of collodion membranes, the pore sizes of which are smaller than the pores in pit membranes.

The impact of a plug in a vascular bundle can be evaluated in relation to the vascular network by means of Poiseuille's law. Dimond and Edgington (13) concluded from such a study that a reduction in half of the effective radius of an unbranched bundle requires a sixteenfold increase in driving pressure if the supply of water to tissues is to remain unchanged. However, a comparable decrease in a stem bundle that forms a part of the vascular network requires an increase in pressure of only a few per cent if the flow rate remains constant. Thus a block in an unbranched bundle has more than 500 times the effect of a comparable block in a bundle that is a part of the network. Moreover, the dimensions of small bundles in the stem and of petiolar bundles are far smaller than those of a large bundle in the stem. Thus the amount of material required to reduce the effective radius to half its former value is much less in an unbranched bundle than in a branching bundle.

In the infected plant, a number of objects may act as physical impediments to flow of water. The first and most obvious of these is the mycelium itself. Waggoner and Dimond (46) made physical measurements of the amount of interference imposed by mycelium in xylem, employing a scale model and hydrodynamic methods. They concluded that 10 hyphae, having a diameter 0.1 that of the containing vessel, interfered with flow so that a sixfold increase in pressure was necessary to maintain the flow rate unchanged. However, considering the number of vessels containing mycelium, they concluded that the net effect on resistance to flow of water is much smaller. Thus the presence of mycelium contributes to, but is not the sole cause of, the observed decrease in rate of flow.

Wilt pathogens, living in vessels, produce spores or bud cells, which become dislodged and move with the transpiration stream to a narrow orifice above (2). The extent of interference with flow of water caused by these fungal cells depend upon how frequently they are formed and where they lodge. Our information on this is scanty at the present time.

Other impediments to flow arise from the host in response to the pathogen. Tyloses frequently arise in wilt diseases and their effect is to reduce the conductive capacity of the vessel. Struckmeyer, *et al.* (42) have demonstrated that sufficient tyloses and gum formation occur in some oaks infected with the oak wilt fungus to account for their high resistance to flow. As decomposition of host cells progresses with increasing amounts of disease, detritis may be freed from cells and ac-

cumulate in vessel openings. Any hydrolytic products of high molecular weight, resulting from cellulase or endopolygalacturonase activity, contribute to gel formation which further impedes the flow. Finally, as resistance to flow increases, the tension in water conducting columns increases to such an extent that, in herbaceous plants at least, heavily infected vessels collapse in response to the tension.

The pathogen, living in the xylem, has a restricted number of ways of increasing the resistance of xylem to the flow of water. To assume a role of extracellular hydrolytic enzymes is reasonable, particularly in view of the ability of many of the wilt-inducing fungi to produce these enzymes *in vitro*. Excellent summaries of the hydrolytic enymes involved in maceration and disintegration of plant tissues have been prepared by Husain and Kelman (22) and by Wood (50, 51).

Some confusion has arisen in phytopathological literature over variations in the names of the various pectic enzymes produced by wilt-inducing fungi. Pectin is composed of galacturonic acid units, united in α-glucosidic linkages to form a long chain in which the carboxyl groups have been partially esterified by methanol. Pectin methyl esterase or pectin esterase hydrolyses the ester linkages, freeing methanol and carboxyl groups. The α-glucosidic linkages are hydrolyzed by the polygalacturonases, some attacking native pectins, whereas others hydrolyze them only when pectin methyl esterase is also present. Some of the polygalacturonases attack the chain from the end, whereas others break the chain at random, liberating large molecules. Depolymerase is of the latter type, but no galacturonic acid is freed as such. The end point of polygalacturonase activity *in vitro* is galacturonic acid.

Fusarium produces pectin methyl esterase and a depolymerase of the endopolygalacturonase type, according to the classification of Demain and Phaff (8). Thus the depolymerase does not free galacturonic acid and hydrolyses pectin rapidly in the presence of pectin methyl esterase (6, 7, 47). *Fusarium* also produces cellulase, both the C_1 enzyme, which attacks native cellulose (21) and the C_x enzyme which hydrolyses carboxylmethyl cellulose (7, 21). The *Verticillium* attacking tomato produces polygalacturonase, very little pectin methyl esterase, and no depolymerase in culture (38).

What evidence exists that these enzymes occur in infected plants? Pectin methyl esterase of fungal origin occurs in infected tomato plants (47). The enzyme produced by the fungus is inactivated by surface-active agents, whereas the pectin methyl esterase of the host is not. More recently Deese and Stahmann (6) have reported higher quantities

of pectin methyl esterase in infected plants than in healthy ones. Wood also (52) detected a higher pectin methyl esterase activity in sap from *Verticillium*-infected tomato plants than in healthy sap and also demonstrated a higher activity in non-turgid healthy plants than in turgid ones. Thus enzyme activity *per se* is meaningful in relation to pathogenesis only if it is characteristic of the combination of host and pathogen or if the activity can be shown to be of fungal origin.

The presence of pectin depolymerase or of polygalacturonase in infected plants is less firmly established. Waggoner and Dimond (47) failed to find enzyme activity in convincing quantities in xylem exudate from diseased plants. Wood (52) failed to find it in *Verticillium*-infected plants, although inactivators in stem tissue may have destroyed the enzyme. Recently, Deese and Stahmann (6, 7) have reported finding abundant amounts of depolymerase in sap from ground stem tissue of *Fusarium*-infected plants but not in healthy sap. Their technique of assay differed from that previously employed, having been based on the appearance of reducing groups, rather than reduction in viscosity.

A number of investigators have assumed that an array of pectic and cellulolytic enzymes is present in infected plants. Let us also make this assumption. Then a number of attractive hypotheses can be set up to account for how wilting occurs.

Although some plants characteristically develop tyloses in response to injury, others do not. Yet tyloses commonly develop in wilt-diseased plants. A tylosis is a ballooning into the vessel lumen of the pectic middle lamella, which is exposed at pits. Contents of adjacent parenchyma cells may move into the tylosis. Pits in vessels are ordinarily bordered, and the torus acts as a valve against the border to prevent tylosis formation. The torus and pit border frequently do not form perfectly so that valve action cannot occur. The action of pectic enzymes upon the torus and pit membrane weakens them structurally, and when a high tension develops in the vessel as a result of growing water shortage in leaves, tyloses may develop more frequently than in healthy plants. Further action of pectolytic enzymes may cause rupture of the tylosic wall, allowing the contents of the former parenchyma cell to escape into the vessel lumen.

Action of depolymerase or of cellulase must occur under unusual conditions. Water flows through the vessel at a measureable speed. The extracellular enzymes, unless liberated in contact with substrate, are likely to be swept with the transpiration stream without ever finding a substrate. But their molecular weight is large, large enough to be considered in the plugging picture. When enzymes are liberated

adjacent to their substrate, they may be adsorbed to it and escape the action of the moving water stream. Then they act by splitting off polysaccharides of large molecular weight and these are swept away with the transpiration stream to a point where they lodge.

The molecular weight of such particles is critical for producing dysfunction. If they are very small, they may move out of the vascular bundles without being trapped. If they are of moderate size, they move to successively finer capillary orifices, higher in the plant, found in leaf veinlets. The studies of Hodgson, Peterson, and Riker (20) are illuminating in defining the molecular sizes that act in this way. Using a series of polyethylene glycols that ranged in molecular weight from 1,540 to 9,000, they demonstrated that each caused wilting in tomato cuttings. The major quantity of these particles was recovered from wilted leaf margins, but as molecular weight increased, the amount trapped in leaf margins decreased, whereas the amount recovered from nonwilted leaflet centers increased. Molecules of these sizes, while recoverable from stem and petiolar tissue, tended to move for the most part into leaf tissue. Polyvinyl alcohol, ranging in molecular weight from 11,500 to 52,000, was also used. Molecules having an average weight of 11,500 and 40,000 induced wilting primarily of leaflets, whereas preparations of high molecular weight, averaging 52,000, caused flaccidity of petioles and stems.

The molecular weight of pectic compounds has been estimated frequently and values range from as high as 200,000 to as low as 16,000, depending upon the source, its method of preparation, and the method of estimation employed (27). Thus, even after splitting of pectic molecules, the hydrolytic product is sufficiently large to lodge in stem tissue and cause wilting. Consider, now, that the depolymerase from *Fusarium* liberates particles with free carboxyl groups. In the presence of Ca or Mg ions, these will form the corresponding salts, which are gels and have a higher molecular weight than the particle liberated by the enzyme. Gel formation in vessels is an effective means of increasing resistance to flow of water through vascular tissue (2, 42).

In considering the mechanisms by which resistance to flow of water is increased during infection, we will find it useful to return to a consideration of various mechanisms in relation to Poiseuille's law. This law states that the pressure head p required to cause a fluid of viscosity η to flow at a rate of v ml./sec. through a capillary of radius r and length l is represented by the following equation:

$$p = \frac{8 \, l \, v \, \eta}{\pi \, r^4}$$

Some investigators have suggested that the hydrolytic products of pectic enzymes increase the viscosity of tracheal fluid, that this is the basis of wilting. We know that the resistance to flow is increased from ten to twentyfold in infected vascular tissue as compared with healthy tissue. Poiseuille's law tells us that to increase the resistance by this amount requires an increase in viscosity of tracheal fluid from ten to twentyfold. Waggoner and Dimond (46) measured the viscosity of tracheal fluid from infected plants and found it to be the same as that of healthy plants. Not enough material is liberated to alter the viscosity of tracheal fluid. Pectin solutions having a viscosity equal to that of water at 10° C will induce wilting of tomato cuttings (14). Clearly, altered viscosity of tracheal fluid is not a major factor in wilting induced by fungal pathogens.

A small change in the effective radius of a capillary causes a large change in its conductive capacity under a constant pressue head. Thus reduction of the effective radius of vascular bundles to 0.563 or 0.474 of their initial value increases their resistance to flow ten to twentyfold. We can see the occluding material: mycelium, tyloses, cellular detritis, and gums. The summed impact of these occlusions may well reduce the effective radius of vessels to the required extent.

When vascular dysfunction occurs, lateral flow of water from one conductive element to another becomes important. Staggered saw cuts half way through the trunk of a tree do not cause it to wilt, and Scholander, *et al.* (40, 41) have provided us with an explanation for this. A similar situation exists when vessels become plugged in wilt diseases. The pores in pit membranes then serve as transport channels laterally to another conductive element. The pores in pit membranes are very small but pits are abundant. This system, although offering higher resistance to flow than the vessels, becomes important when vascular dysfunction occurs.

When large molecules are carried in the transpiration stream, they gradually plug the pores in pit membranes and lateral transfer of water ceases. The effect on flow at this stage is devastating. The vascular system can be regarded as an ultrafilter from which particulate matter is removed and from which even large molecules are removed before the water in which they are carried arrives at the leaf parenchyma.

The role of polyphenols in vascular discoloration.—Vascular discoloration is characteristic of a number of wilt diseases. In *Fusarium*-infected tomatoes, the first discoloration appears in xylem parenchyma, adjacent to conductive xylem. Gradually, the dark-colored pigments

in these cells appear in vessels. The discoloration is caused by brown to black melanoid pigments. Under the influence of polyphenol oxidase, polyphenols are oxidized to quinones, and these polymerize to form dark-colored melanins. Davis, *et al.* (5) showed that *Fusarium* can grow on a glycoside as a sole source of carbon and demonstrated that it produces a β-glucosidase that liberates glucose, which the fungus then uses in carbon assimilation. This β-glucosidase was also found in *Fusarium*-infected but not in healthy tomato plants. These workers suggested that pectic enzymes produce mild maceration of the middle lamella at pits in vessels, after which β-glucosidase enters xylem parenchyma. In the parenchyma the β-glucosidase frees phenols from phenolic glucosides and the resulting phenols are then oxidized by polyphenol oxidase in the parenchyma cell. Melanin formation then ensues.

Waggoner and Dimond (48) examined the possibility that free phenols occur in the tomato stem tissues from which melanins might arise more simply. Though they found polyphenol oxidase, they found no chlorogenic acid or other free phenols, and concluded that the phenolic substrates for melanin formation are not in free form in the stem.

Recently Mace (33) has demonstrated the presence of quantities of 3-hydroxytyramine in scattered parenchyma cells surrounding xylem vessels in banana roots and showed that as this phenol disappears through oxidation and polymerization, melanin pigments appear, causing initial vascular discoloration. *Fusarium*, though largely confined to xylem vessels, escapes from them into parenchyma occasionally. The ensuing disorganization of the cell is sufficient to set off the melanin reaction.

Two interactions involving the polyphenol oxidases and wilt diseases remain to be discussed. Fusaric acid has proven to be a competitive inhibitor of polyphenol oxidase (3). Apparently it is sufficiently active as an inhibitor to affect enzyme activity in the discussed plant, though this has not yet been demonstrated *in vivo*.

Phenolases and polygalacturonases also interact with one another. Deverall (9) and Deverall and Wood (10) have shown that in *Botrytis* infections, the maceration produced by polygalacturonase liberates the phenolases and this initiates the melanin reaction. The resulting melanoid pigments inactive polygalacturonase. The phenolases were activated by pectic materials and by carboxymethyl cellulose. This work suggests an interaction in wilt diseases that may explain why the pectolytic enzymes have been difficult to detect in infected plants. Further interactions between the two symptoms may affect the extent of symptom development.

The wilt diseases offer intriguing opportunities for further investigation. Much progress has been made toward understanding them, yet much remains to be done. Let us hope that when West Virginia University celebrates its hundredth year of plant pathology the wilt diseases will have ceased to take a heavy toll our crops and that plant pathologists will then be able to control them efficiently.

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A Brief Summation of the Symposium Papers

J. G. Leach

From Dr. Lilly's paper we may conclude that:

1. If we are to understand the physiology of fungus diseases, it is important to know more about the physiology of fungi grown in pure culture on synthetic media under carefully controlled conditions.

2. The available information on the physiology of fungi indicates that fungi are extremely variable and that their physiology is greatly modified by slight changes in composition of media and the various factors of the environment.

3. Because of this variability, it is difficult, on the basis of present information, to make many broad generalizations about the physiology of growth and reproduction of fungi.

4. Great care is necessary in measuring growth of fungi. The dry weight of the mycelium appears to be the most accurate measure of growth.

From Dr. Barnett's presentation we learn that:

1. More knowledge and understanding of the phenomenon of parasitism is essential to the solution of the problems of disease physiology.

2. Types of mycoparasitism occur that closely parallel the recognized types of parasitism by fungi on higher plants.

3. Mycoparasitism, because of our ability to control the nutrients available to both host and parasite, offers a new and promising approach to the study of parasitism.

4. A growth factor is produced by many fungi which in high dilutions enables certain mycoparasites, normally biotrophic in nature, to grow in axenic culture. Further study of this factor should pave the way to a better understanding of the so-called "obligate parasites" such as the rusts and mildews.

5. High susceptibility of certain host fungi is correlated with high concentration of soluble nitrogen in their mycelium and with an excess of microelements in the medium.

6. The degree of susceptibility to certain mycoparasites may be related to the presence of lipid compounds.

From Dr. Dimond's paper it is evident that:

1. The mechanism of wilt production is a complicated phenomenon. It is evident that a dysfunction of the xylem is involved, but the physiological processes that lead to dysfunction are not clearly understood. A number of theories have been offered, none of which explains satisfactorily all of the observed syndrome. Fusaric acid produced by certain species of *Fusarium* has been suggested as the active principle and that its chelating action robs the host cells of iron and probably other essential metals.

2. Another theory holds that the xylem dysfunction is the result of the action of pectic enzymes produced by the wilt pathogens. This theory holds that the hydrolytic products of the pectic enzymes are composed of large particles that plug the pores in the pit membrane and inhibit vascular flow even though the viscosity of the tracheal fluid may not be greatly increased.

3. The problem is further complicated by the production of certain growth stimulating compounds such as ethylene in wilting plants and by the chemical changes involved in the characteristic browning of vascular elements in wilted plants.

4. It is evident that much progress has been made in the study of the physiological processes involved in wilt production but much more work with improved biochemical techniques will be necessary before we can gain a satisfactory explanation of the mechanisms involved.